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A Comparative Study of the Diagnostic Performance of Amniotic Fluid Glucose, White Blood Cell Count, Interleukin-6, and Gram Stain in the Detection of Microbial Invasion in Patients With Preterm Premature Rupture Of Membranes

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Abstract

OBJECTIVE: Our aim was to compare the value of amniotic fluid tests for the detection of microbial invasion of the amniotic cavity and in the prediction of the amniocentesis-to-delivery interval and neonatal complications in patients with preterm premature rupture of membranes.

STUDY DESIGN: Amniotic fluid was obtained by transabdominal amniocentesis from 110 consecutive patients with preterm premature rupture of membranes. Fluid was cultured for aerobic and anaerobic bacteria, as well as mycoplasmas. Amniotic fluid analysis included a Gram stain examination, white blood cell count, and glucose and interleukin-6 determinations. Logistic regression and survival techniques (proportional hazards model) were used for statistical analysis.

RESULTS: (1) The prevalence of positive amniotic fluid cultures in patients with preterm premature rupture of membranes was 38% (42/110); (2) patients with microbial invasion had a shorter amniocentesis-to-delivery interval and a higher neonatal complication rate than patients with negative cultures; (3) the most sensitive test for the detection of microbial invasion of the amniotic cavity was amniotic fluid interleukin-6 determinations (cutoff 7.9 ng/ml) (sensitivity: for IL-6 80.9%; for white blood cell count 57.1%; for glucose 57.1%; for Gram stain 23.8%; $p < 0.05$ for all comparisons); (4) the most specific test for the detection of microbial invasion was the Gram stain of amniotic fluid (specificity: for Gram stain 98.5%; for white blood cell count 77.9%; for interleukin-6 75%; for glucose 73.5%; $p < 0.01$ for all); (5) of all amniotic fluid tests, interleukin-6 determination was the only test that had significant clinical value in the prediction of the amniocentesis-to-delivery interval and neonatal complications.

CONCLUSION: Interleukin-6 concentrations in amniotic fluid are a better predictor of microbial invasion of the amniotic cavity, amniocentesis-to-delivery interval and neonatal complications than the amniotic fluid Gram stain, glucose, or white blood cell count in patients with preterm premature rupture of membranes. (AM J OBSTET GYNECOL 1993;169:839-51.)

Key words: Interleukin-6, amniotic fluid, Gram stain, preterm labor, cytokines, preterm premature rupture of membranes, white blood cell count, glucose concentration

Microbial invasion of the amniotic cavity is present in approximately one third of patients with preterm premature rupture of membranes [1,2,3,4,5,6,7,8,9]. An early diagnosis of this condition is important because neonates born to mothers with microbial invasion are at high risk for infection-related and non-infection-related complications [1,2,3,5]. Regrettably, clinical chorioamnionitis occurs late during the course of this disease and the results of the microbial cultures are often not available in time for important patient management decisions. Therefore there has been considerable interest in the development of rapid tests for the diagnosis of microbial invasion of the amniotic cavity by analysis of amniotic fluid. These tests include Gram stain, [1,2,3,4,5,6,7,9] white blood cell count, [8,10] glucose concentrations, [9,11,12] leukocyte esterase activity, [9,13,14] leukoattractants, [15] and interleukin-6 (IL-6) concentrations [16,17]. The value of these tests has been examined in patients with preterm labor and intact membranes and in patients with preterm premature rupture of membranes. However, there is a paucity of data about the comparative diagnostic and prognostic value of these tests in patients with preterm premature rupture of membranes.

The purpose of this study was to compare the diagnostic performance of these tests in the detection of microbial invasion of the amniotic cavity and in the prediction of the duration of pregnancy and neonatal outcome in patients with preterm premature rupture of membranes. Moreover, this study also compares the diagnostic performance of these tests in patients with preterm labor with intact membranes and preterm premature rupture of membranes.

Material and methods

Patient population. The study population consisted of patients admitted with the diagnosis of preterm premature rupture of membranes, who had singleton gestations, and who underwent amniocentesis for the assessment of the microbiologic status of the amniotic cavity. Samples were collected between January 1991 and August 1992 at Yale--New Haven Hospital and Hutzel Hospital, Wayne State University. Patients and results of the amniotic fluid tests included in this study have not been used in our previous studies. Premature rupture of the membranes was diagnosed by sterile speculum examination confirming pooling of amniotic fluid in the vagina, a positive Nitrazine test result, and a positive ferning test result. Tocolysis and steroids were not used in patient management.

Retrieval of amniotic fluid. In all cases, amniotic fluid was retrieved by transabdominal amniocentesis, which was performed under ultrasonographic guidance. Cultures for mycoplasmas and aerobic and

anaerobic bacteria, as well as amniotic fluid white blood cell counts and Gram stains, were performed immediately after collection. The remaining amniotic fluid was centrifuged at 700g for 10 minutes at 4 degrees C, divided into units of 300 microliters per vial, and stored in polypropylene tubes at -70 degrees C until assayed for glucose and IL-6. Samples were not subjected to freeze-thaw cycles before being assayed.

Microbiologic culture technique. Amniotic fluid was transported to the laboratory in a capped plastic syringe immediately after collection. Plating occurred within 30 minutes of collection. Amniotic fluid was cultured for aerobic and anaerobic bacteria and for mycoplasmas by means of methods previously described in detail [18]. The culture for mycoplasmas was used for clinical management in only one case.

Gram stain examination. Gram stain examination was performed in all samples with commercial reagents (crystal violet, safranin, and Gram's iodine; Difco Laboratories, Detroit) under standard conditions [6]. Stained slides were examined by trained technologists, and the presence or absence of microorganisms was noted. The results of the Gram stain examinations were communicated to the clinicians. Patients with a positive Gram stain examination of amniotic fluid for bacteria were given parenteral antibiotics and delivered.

Amniotic fluid white blood cell count. An aliquot of amniotic fluid was transported to the hematology laboratory and examined in a hemocytometer chamber (Neubauer ruled) for the presence of white blood cells, as previously described [10]. The results were not available to the clinicians.

Amniotic fluid glucose determinations. Glucose analysis was performed by the glucose oxidase method with a Beckman glucose analyzer as previously described [11]. All samples were run in duplicate. The coefficient of variation was 5%.

Amniotic fluid IL-6 determinations. IL-6 levels in amniotic fluid were measured by two-site enzyme-linked immunoassay (ELISA) with two monoclonal antibodies (7IL6-H12 and 5IL6-H17), which bind nonoverlapping epitopes on IL-6. Monoclonal antibodies to IL-6 were developed and used as previously described [19,20,21]. ELISA conditions were optimized and the assay was performed with a 25 microliters standard or sample volume in a flat-bottom half-area immunoassay-radioimmunoassay microplate (Costar, Cambridge, Mass.). The National Institute for Biological Standards and Control interim reference IL-6 (88/514) was used as the standard in the immunoassay. This ELISA detects not only free monomeric IL-6 but also IL-6 complexes found in human body fluids [21]. The ELISA format was adjusted to give a lower cutoff of 0.1 ng/ml using the IL-6 standard, and the coefficients of variation were typically <5% for concentrations of standard from 0.082 to 20 ng/ml. Amniotic fluid samples were centrifugated at 700g for 10 minutes

to remove insoluble material and the supernatant was assayed in duplicate. Samples containing >20 ng/ml of IL-6 were reassayed at greater dilution. For amniotic fluid samples, the intraassay and interassay coefficients of variation were 6.4% and 7.9%, respectively. Recovery of exogenously added IL-6 was 101% +-\5% for 13 individual amniotic fluid samples spiked with 2.2 ng/ml IL-6 and 100% +-\1% for a mixture of five amniotic fluid samples spiked with different amounts of IL-6 in the concentration range 0.027 to 20 ng/ml.

Criteria for diagnosis of chorioamnionitis and neonatal morbidity. Clinical chorioamnionitis was defined according to the criteria proposed by Gibbs et al [22]. The diagnosis required a temperature elevation to 37.8 degrees C and two or more of the following criteria: uterine tenderness, malodorous vaginal discharge, and leukocytosis. Leukocytosis was defined as a white blood cell count >15,000/mm^3. Neonatal sepsis was diagnosed in the presence of a positive culture of blood, urine, or cerebrospinal fluid. Possible neonatal sepsis was diagnosed in the absence of a positive culture when two or more of the following criteria were present: white blood cell count of <5000/mm^3, polymorphonuclear leukocyte count of <1800/mm^3, I/T ratio (ratio of bands to total neutrophils) >0.2, or a positive gastric aspirate for polymorphonuclear leukocytes showing >5 white blood cells per high-power field. These criteria have been previously used in the pediatric and obstetric literature [23,24,25]. The diagnosis of respiratory distress syndrome (RDS) required the presence of respiratory grunting and retracting, an increased oxygen requirement (FIO_2 >0.4), and diagnostic radiographic and laboratory findings in the absence of evidence of other causes of respiratory disease. Pneumonia was diagnosed in the presence of definite clinical and radiologic findings, with or without a positive culture from tracheal aspirate, blood, or chest tube specimen. Bronchopulmonary dysplasia was diagnosed if the neonate required more than 60% oxygen for a day or more and prolonged ventilatory therapy with constant positive airway pressure, had typical radiographic changes, or had dysplasia of the bronchopulmonary tree at autopsy. Intraventricular hemorrhage was diagnosed by ultrasonographic examination of the neonatal head. Necrotizing enterocolitis was diagnosed in the presence of abdominal distention and feeding intolerance for at least 24 hours (vomiting or increased gastric residual) with clear radiologic evidence of intramural air, perforation, meconium plug syndrome, or definite surgical or autopsy findings of necrotizing enterocolitis.

Statistical analysis. Either a Mann-Whitney U test or Student's t test was used for comparison of continuous variables. Comparisons of proportions were performed with chi squared (chi^2) test or Fisher's exact test. The relationship between the results of amniotic fluid white blood cell count, glucose concentrations, and IL-6 concentrations was analyzed by Spearman's rank correlation. Receiver-operator characteristic curves were constructed to describe the relationship between the sensitivity

(true-positive rate) and the false-positive rate for different values of amniotic fluid white blood cells, glucose, and IL-6 in the detection of a positive amniotic fluid culture. Diagnostic indices (sensitivity and specificity) and positive and negative predictive values were calculated for the Gram stain, amniotic fluid white blood cell count, glucose, IL-6, and various combinations of these tests in the diagnosis of infection. Comparisons between sensitivity and specificity of the different diagnostic modalities were performed with a modified t test for correlated samples, as described by Galen and Gambino [26].

Logistic regression was used to investigate the relationship between a number of different response variables and various predictors. The response variables were the results of amniotic fluid culture and the occurrence of neonatal complications. The explanatory variables were the results of amniotic fluid Gram stain, white blood cell count, glucose and IL-6 concentrations. The regression relationship between the log odds of a positive amniotic fluid culture and the explanatory variables was examined using logistic regression and a stepwise covariate selection procedure. The results of the Gram stain were first entered into the model followed by the results of the other amniotic fluid tests. Then, similar analyses were repeated after the order of entering these covariates into the model was changed. This analysis was designed to identify the most parsimonious model that adequately explained the data. Similar analyses were carried out to model the log odds of the occurrence of neonatal complications.

Cox's proportional hazards modeling was used to examine the relationship between amniocentesis-todelivery interval and a number of covariates such as amniotic fluid IL-6 and glucose concentrations, gestational age at admission, cervical dilatation, amniotic fluid white blood cell count, and Gram stain. Women who did not go into labor spontaneously, because they were delivered for maternal or fetal indications, were treated as censored observations with a censoring time equal to the amniocentesis-to-delivery interval. The generalized Wilcoxon test was used to determine the amniocentesis-to-delivery interval in patients with normal and abnormal rapid test results.

Results

Characteristics of population studied, obstetric and neonatal outcome. One hundred ten patients were included in the study. The prevalence of positive amniotic fluid culture was 38% (42/110). Table I describes the clinical characteristics of patients according to amniotic fluid culture results. Although there was no difference in the mean gestational age at admission between patients in these two groups, patients with a positive amniotic fluid culture had a significantly lower gestational age at delivery and a shorter admission-to-delivery interval than patients with a negative culture Figure 1. The prevalence of significant neonatal morbidity (defined as proven sepsis, probable sepsis, intraventricular hemorrhage, respiratory distress syndrome,

pneumonia, bronchopulmonary dysplasia, or necrotizing enterocolitis) and mortality was significantly higher in patients with positive amniotic fluid cultures than in those with a negative amniotic fluid culture.

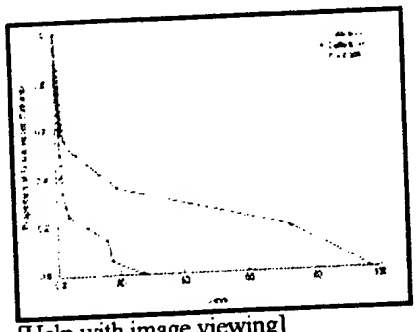

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Figure 1. Survival analysis of amniocentesis-to-delivery interval in patients with preterm premature rupture of membranes according to amniotic fluid culture results. Patients with a positive amniotic fluid culture had significantly shorter median amniocentesis-to-delivery interval than did patients with a negative culture (median 15 hours, range 0.5 to 550 hours vs median 56 hours, range 1 to 2450 hours; $p < 0.005$, generalized Wilcoxon test for survival analysis). Patients with a positive Gram stain were considered to have censored observations

Amniotic fluid white blood cell count, glucose, and IL-6 in patients with and without microbial invasion of amniotic cavity. Patients with positive amniotic fluid cultures for microorganisms had significantly higher median amniotic fluid white blood cell counts and IL-6 concentrations but lower median glucose concentrations than did patients with negative amniotic fluid cultures Figure 2.

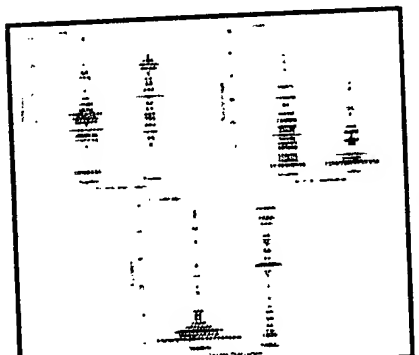

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Figure 2. Amniotic fluid white blood cell count (WBC/mm^3), glucose concentrations (mg/dl), and IL-6 concentrations (ng/ml) according to amniotic fluid culture results. Patients with positive amniotic fluid cultures for microorganisms had significantly higher median amniotic fluid white blood cell count and median IL-6 concentrations but lower median glucose concentrations than did patients with negative amniotic fluid cultures (white blood cell count: median 10.5, range 0 to 8200 cells/mm^3 vs median 78.5, range 1 to 1740 cells/mm^3, $p < 0.0005$; glucose concentration: median 14.75, range 0 to 48.5 mg/dl vs median 4.5, range 0 to 42.33 mg/dl, $p < 0.005$; IL-6 concentration: median 3.8, range 0.24 to 46.69 ng/ml vs median 26.8, range 0.84 to 47.15, $p < 0.000001$, Mann-Whitney U test)

There was a significant correlation between the results of amniotic fluid white blood cell count, glucose concentrations, and IL-6 concentrations (white blood cell count vs glucose concentration: $r = -0.48$, $p < 0.000001$;

white blood cell count vs IL-6 concentration: $r = 0.54$, $p < 0.000001$; glucose concentration vs IL-6 concentration: $r = -0.27$, $p < 0.005$; respectively, Spearman's rank correlation test). The correlation between amniotic fluid IL-6 and amniotic fluid white blood cell count was higher than that between amniotic fluid IL-6 and glucose determinations ($r = 0.54$ vs $r = -0.27$, $p < 0.05$).

Receiver-operator characteristic curve analysis of amniotic fluid white blood cell count, glucose, and IL-6 in diagnosis of microbial invasion of amniotic cavity. Receiver-operator characteristic curve analysis demonstrated that the diagnostic performance of amniotic fluid IL-6 determination was superior to that of amniotic fluid white blood cell count and amniotic fluid glucose level ($p < 0.001$). No differences in the performance of amniotic fluid glucose and amniotic fluid white blood cell count were found ($p > 0.05$) Figure 3. However, the receiver-operator characteristic curves for amniotic fluid white blood cell count determinations, glucose determinations, and IL-6 determinations in patients with preterm premature rupture of membranes were significantly different from those previously published by our group for the same tests in patients with preterm labor and intact membrane Figure 4.

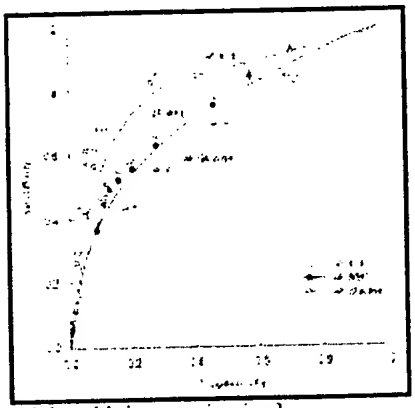

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Figure 3. Receiver-operator characteristic curve analysis of amniotic fluid IL-6 concentrations, amniotic fluid white blood cell count, and amniotic fluid glucose concentrations in diagnosis of positive amniotic fluid culture results (area under curve for amniotic fluid IL-6 concentration: 0.80, SE 0.05, $p < 0.000001$; for amniotic fluid white blood cell count: 0.70, SE 0.05, $p < 0.000005$; for amniotic fluid glucose concentration: 0.68, SE 0.05, $p < 0.0005$; respectively)

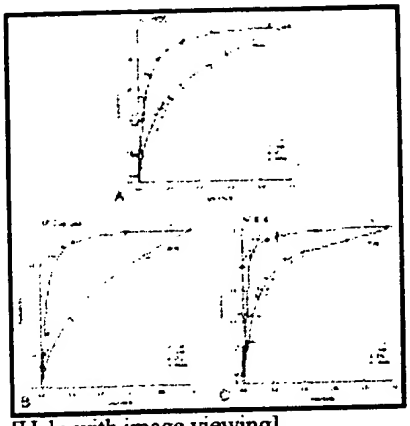

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Figure 4. Comparison of receiver-operator characteristic curves in patients with preterm premature rupture of membranes (PROM) and in patients with preterm labor (PTL) with intact membranes in diagnosis of positive amniotic fluid culture results. Receiver-operator characteristic curves for amniotic fluid white blood cell count determinations, amniotic fluid glucose determinations, and amniotic fluid IL-6 determinations in patients with preterm premature rupture of membranes were significantly different from those previously published by our group for the same tests in patients with preterm labor and intact membranes (area under curves for amniotic fluid white blood cell count: 0.70 vs 0.89, z = 2.86; for amniotic fluid glucose determination: 0.68 vs 0.93, z = 4.08; for amniotic fluid IL-6 determination: 0.80 vs 0.97, z = 3.26; respectively; $p < 0.05$ for all)

Diagnostic indices of amniotic fluid tests in detection of microbial invasion of amniotic cavity. As with any other diagnostic test, there is a tradeoff between the sensitivity and the false-positive rate (100 - specificity). We chose to use a cutoff at 30 cells/mm^3 for amniotic fluid white blood cell count, 10 mg/dl for amniotic fluid glucose level, and 7.9 ng/ml for amniotic fluid IL-6, because these values seemed to represent a reasonable compromise between a true-positive rate and a false-positive rate. We have previously published the diagnostic indices of amniotic fluid white blood cell counts and amniotic fluid glucose with cutoffs of 50 cells/mm^3 and 14 mg/ml in patients with preterm labor with intact membranes; therefore we have also included the diagnostic indices with these cutoff levels.

Table II displays the diagnostic index values of four different tests in the detection of the positive amniotic fluid culture. Amniotic fluid IL-6 determinations had the highest sensitivity (80.9%, 34/42) in the detection of positive amniotic fluid cultures of all tests performed (amniotic fluid white blood cell count determinations: 57.1% (24/42); amniotic fluid glucose determinations: 57.1% (24/42); Gram stain: 23.8% (10/42); $p < 0.05$ for all comparisons). The Gram stain had the highest specificity (98.5% (67/68)) of any test (amniotic fluid white blood cell count: 77.9% (53/68); amniotic fluid glucose determinations: 73.5% (50/68); amniotic fluid IL-6 determinations: 75% (51/68); $p < 0.01$ for all comparisons).

When the Gram stain and the other three tests were used in combination (and the presence of any abnormal test result considered indicative of microbial invasion), the combined use of all four of these different tests had the highest sensitivity (92.9%, 39/42) in the detection of a positive amniotic fluid culture. The combined use of the Gram stain and amniotic fluid white blood cell determinations showed the highest specificity (77.9%, 53/68) Table II.

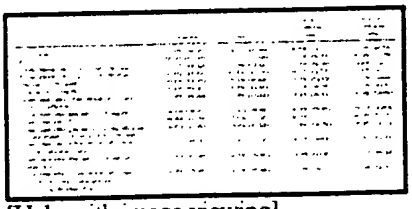

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Table II. Diagnostic indices and predictive values of different amniotic fluid tests in the detection of positive amniotic fluid culture

Logistic regression analysis of value of different amniotic fluid tests and amniotic fluid culture results. Logistic regression revealed a significant relationship between the log odds of results of amniotic fluid culture and Gram stain (chi squared (chi^2) = 14.99, $p < 0.0005$), amniotic fluid white blood cell determinations (chi squared (chi^2) = 13.92, $p < 0.0005$), amniotic fluid glucose determinations (chi squared (chi sup 2) = 10.33, $p < 0.005$), and amniotic fluid IL-6 determinations (chi squared (chi^2) = 34.53, $p < 0.000001$). However, amniotic fluid IL-6 concentration was the only covariate that was independently significant (amniotic fluid IL-6 determinations: $p < 0.0001$; amniotic fluid glucose determinations: $p = 0.06$; Gram stain: $p = 0.15$; amniotic fluid white blood cell count: $p = 0.35$).

Stepwise covariate selection procedure indicated that when amniotic fluid IL-6 concentrations were entered first into the model, only the addition of glucose concentrations was statistically significant (improvement in chi squared (chi^2): amniotic fluid glucose determinations, 5.85, $p < 0.05$; amniotic fluid white blood cell count, 3.70, $p = 0.053$; Gram stain, 3.38, $p = 0.084$). Further, when the other covariates were entered first into the model, the results of amniotic fluid IL-6 concentrations showed a statistically significant increment in the chi squared (chi^2) (improvement in chi squared (chi^2) ranged from 17.8 to 30.1, $p < 0.01$). When amniotic fluid IL-6 concentrations were omitted from the model, all three remaining covariates retained statistical significance.

Maternal and fetal indications for delivery. Seven patients were delivered because of maternal indications (placenta previa, chorioamnionitis, genital herpes) and 37 were delivered because of fetal indications (mature lecithin/sphingomyelin ratio, positive Gram stain, and fetal distress). These cases were considered as censored observations for survival analysis.

Characteristics of patients with "false-positive" amniotic fluid test results. Seventeen patients had an amniotic fluid IL-6 concentration >=7.9 ng/ml and a negative amniotic fluid culture. Of these 17 patients, eight (47%) were delivered for maternal or fetal indications. Of the remaining nine patients, eight of them were delivered within 72 hours of amniocentesis. The remaining patient delivered spontaneously approximately 5 days after

the amniocentesis; however, she had clinical and histologic chorioamnionitis and the neonate was diagnosed as having sepsis. Eighty-seven percent of patients (13/15) showed histologic evidence of chorioamnionitis. Two neonates died immediately after birth. In addition, two neonates had documented sepsis, and another five were diagnosed as having presumed sepsis.

Fifteen patients had an amniotic fluid white blood cell count >=30 cells/mm^3 and a negative amniotic fluid culture. Of these 15 patients, seven (46.7%) were delivered for maternal or fetal indications. Of the remaining eight patients, six were delivered within 72 hours of amniocentesis. Eighty-seven percent of patients (13/15) showed histologic evidence of chorioamnionitis. Two neonates died immediately after birth, and one neonate was diagnosed as having presumed sepsis.

Eighteen patients had an amniotic fluid glucose concentration <10 mg/dl and a negative amniotic fluid culture. Of these 18 patients, seven (38.9%) were delivered for maternal or fetal indications. Of the remaining 11 patients, delivery occurred <72 hours after the amniocentesis in 10. None had clinical signs of chorioamnionitis. Fifty-three percent of patients (8/15) showed histologic evidence of chorioamnionitis. One neonate died immediately after birth, and three neonates were diagnosed as having presumed sepsis.

Among patients with positive amniotic fluid cultures, 19% (8/42) had polymicrobial infections. Pure mycoplasmal infections were present in 62% of patients (26/42). Median amniotic fluid white blood cell count, glucose concentrations, and IL-6 concentrations were not different in patients with positive amniotic fluid cultures only for mycoplasmas and those with positive cultures for nonmycoplasmas Table III. However, patients with positive amniotic fluid cultures for bacteria and large colony counts (>100,000 colony-forming units per milliliter) had significantly higher median amniotic fluid white blood cell counts, lower median glucose concentrations, and higher IL-6 concentrations than did patients with positive cultures for bacteria and small colony count (<100,000 colony-forming units per milliliter) Table IV.

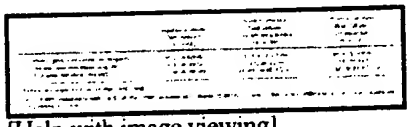

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Table III. Amniotic fluid white blood cell count, glucose concentrations, and IL-6 concentrations of patients with preterm premature rupture of membranes and positive amniotic fluid cultures according to amniotic fluid culture results

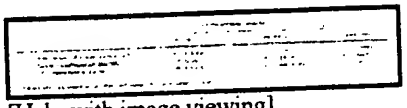

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Table IV. Amniotic fluid white blood cell count, glucose concentrations, and IL-6 concentrations of patients with preterm premature rupture of membranes with positive amniotic fluid cultures for bacteria according to count of bacterial colony-forming units

Clinical chorioamnionitis and neonatal sepsis. Clinical chorioamnionitis occurred in 7.3% of patients (8/110). Five patients had positive amniotic fluid cultures for microorganisms. The remaining three patients had negative amniotic fluid cultures, but all of these patients had amniotic fluid IL-6 concentrations above 7.9 ng/ml. The first patient had chorioamnionitis on admission and labor was induced. The newborn had no evidence of sepsis. In the second patient chorioamnionitis developed approximately 5 days after amniocentesis, and she underwent a cesarean section for fetal distress. The neonate was diagnosed as having presumed sepsis. Placental examination showed acute histologic chorioamnionitis. In the third patient clinical chorioamnionitis developed 12 days after admission. However, the amniotic fluid culture from repeated amniocenteses performed 3 days and 7 days after admission was positive for Ureaplasma urealyticum. In view of the early gestational age (28 weeks), it was decided to continue antibiotic therapy in an effort to prolong the pregnancy. The patient underwent a cesarean section because of breech presentation 12 days after admission. The neonate was diagnosed as having presumed sepsis. The placenta showed severe histologic chorioamnionitis.

Amniotic fluid tests and amniocentesis-to-delivery interval. To examine the relationship between the amniocentesis-to-delivery interval and various amniotic fluid tests, Cox's proportional hazards model was performed in patients admitted without labor (n = 85). The result of amniotic fluid IL-6 determination was a significant predictor of the duration of pregnancy ($p < 0.005$) when we controlled for gestational age Table V. In addition, the amniocentesis-to-delivery interval of patients with high amniotic fluid IL-6 concentration (>=7.9 ng/ml) was significantly shorter than that of patients with low amniotic fluid IL-6 (<7.9 ng/ml) (median 29.5 hours, range 5 to 384 hours vs median 77 hours, range 3.5 to 2450 hours; $p < 0.05$). However, there was no significant difference in amniocentesis-to-delivery interval between the patients with low amniotic fluid glucose concentrations (<10 mg/dl) and those with high amniotic fluid glucose concentrations (>=10 mg/dl) (median 27 hours, range 3.5 to 360 hours vs median 78 hours, range 6 to 2450 hours; $p = 0.085$) or between the patients with high amniotic fluid white blood cell count (>=30 cells/mm^3) and those with low amniotic fluid white blood cell count (<30 cells/mm^3) (median 44.5 hours; range 6 to 2450 hours vs median 53 hours, range 3.5 to 1800 hours; $p > 0.1$, generalized Wilcoxon test).

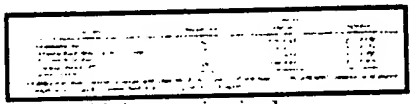

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Table V. Cox's proportional-hazards analysis of amniocentesis-to-delivery interval

Amniotic fluid tests and neonatal morbidity and mortality. Mothers delivered of neonates with significant neonatal morbidity or mortality had a significantly higher amniotic fluid white blood cell count and higher amniotic fluid IL-6 concentrations than mothers delivered of neonates without complications Table VI. The regression relationship between the occurrence of neonatal morbidity-mortality and several covariates that could be known at the time of admission or shortly after was examined using multiple logistic regression. Gestational age at amniocentesis, amniotic fluid IL-6, and Gram stain demonstrated an independent significant relationship with the occurrence of neonatal morbidity and mortality ($p < 0.05$ for each). However, when all of these covariates were simultaneously entered into the model, only the results of amniotic fluid IL-6 concentrations and gestational age at admission retained statistical significance (odds ratio: amniotic fluid IL-6 concentrations, 3.32 $p < 0.05$; gestational age at admission, 0.66 $p < 0.05$; Gram stain, 4.21, $p = 0.09$; amniotic fluid white blood cell count, 0.42, $p > 0.1$; amniotic fluid glucose, 1.32, $p > 0.1$).

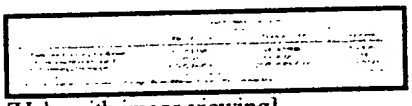

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Table VI. Amniotic fluid white blood cell count, glucose concentration, and IL-6 concentration of patients with preterm premature rupture of membranes according to presence of neonatal morbidity or mortality

Comment

The first finding of our study is that patients with preterm premature rupture of membranes and a positive amniotic fluid culture have a shorter amniocentesis-to-delivery interval than patients with a negative amniotic fluid culture. Since the Gram stain examination of amniotic fluid was used for patient management, it might be argued that induction of labor in these patients may account for this observation. However, the difference remains significant when all patients with a positive Gram stain were removed from the data set. Furthermore, survival analysis of the admission-to-delivery interval in which patients with a positive Gram stain were considered as censored observations yielded similar results Figure 1.

The rate of neonatal complications was higher in patients with microbial invasion of the amniotic cavity than in those without it. These data are in keeping with observations made by other investigators [1,2,3,5]. Collectively these data provide strong evidence about the importance of microbial invasion of the amniotic cavity as a risk factor for adverse pregnancy and

neonatal outcome.

The most common microorganisms isolated from the amniotic fluid were mycoplasmas. This is in keeping with previous observations made in our institution [6,7] and also by Coultrip et al [8] and Gauthier and Meyer [9]. It is noteworthy that patients with preterm premature rupture of membranes have a higher rate of microbial invasion exclusively by mycoplasmas than do patients with preterm labor with intact membranes (61.9%, 26/42 vs 12.5%, 3/24; $p < 0.001$) [27]. Further studies are required to elucidate why there is an excess of microbial invasion exclusively due to mycoplasmas (and in particular by Ureaplasma urealyticum) in patients with preterm premature rupture of membranes. Although mycoplasmas are extremely frequent facultative microorganisms in the lower genital tract, it is unlikely that their mere presence is the explanation for our observation because other prevalent microorganisms (e.g., Lactobacillus sp.) were not found as often in the amniotic cavity of patients in this study.

Our data clearly indicate that microbial invasion of the amniotic cavity with mycoplasmas is capable of eliciting a host response because patients with positive cultures for mycoplasmas had a higher amniotic fluid IL-6 and white blood cell count than did patients with a negative amniotic fluid culture. Moreover, we found no difference in the median amniotic fluid IL-6 and white blood cell count between patients with microbial invasion exclusively by mycoplasmas and that by other aerobic and anaerobic bacteria (see Table III).

A novel observation made during the course of this study is that the inoculum size is an important factor in determining the changes in amniotic fluid glucose, white blood cell count, and IL-6 concentrations. Indeed, patients with more than 10^5 colony-forming units per milliliter had significantly higher amniotic fluid IL-6 and white blood cell count and lower glucose concentrations than did patients with <10 [5] colony-forming units per milliliter. A limitation of this study is that this hypothesis was not tested in the context of microbial invasion with mycoplasmas because quantitative cultures for these microorganisms were not performed.

How to detect microbial invasion of the amniotic cavity? Although all the tests analyzed in this study are of value in the detection of microbial invasion of the amniotic cavity Figure 2, our results clearly show that amniotic fluid IL-6 determinations are better predictors of amniotic fluid culture results, the duration of the admission-to-delivery interval, and neonatal morbidity and mortality than are Gram stain, amniotic fluid white blood cell count, and amniotic fluid glucose. An amniotic fluid IL-6 concentration above 7.9 ng/ml had a sensitivity of 80.9% in the detection of a positive culture but a specificity of 75%. However, the false-positive rate of 25% (100 - specificity) is more apparent than real as virtually all patients delivered within 72 hours and had neonatal complications and histologic chorioamnionitis.

In this study the Gram stain of amniotic fluid had an extremely low sensitivity (23%) in the detection of microbial invasion of the amniotic cavity. The most likely explanation for this is that mycoplasmas, the most common isolates in this study, are not visible on a Gram stain examination. There was only one positive Gram stain in patients with positive amniotic fluid culture with mycoplasmas. This case may represent either a false-positive Gram stain or a false-negative culture for other bacteria.

An interesting finding in this study is that all rapid tests for the detection of microbial invasion of the amniotic cavity performed significantly better in patients with preterm labor and intact membranes than in patients with preterm premature rupture of membranes (Fig. 4; for Gram stain: 23.8%, 10/42 vs 79.1%, 19/24; $p < 0.01$; Table II). Amniotic fluid glucose, white blood cell count, and IL-6 concentrations largely reflect host responses to microbial invasion; therefore we interpret our data as indicating that there are differences in the biologic characteristics of microbial invasion in preterm labor with intact membranes and in preterm premature rupture of membranes. Microbial invasion of recent onset, such as the one that probably occurs after rupture of membranes may be sufficient to lead to a positive culture but not to a robust host response. In contrast, microbial invasion of the amniotic cavity that occurs in patients with intact membranes is of longer duration and thus allows a more robust host response. Evidence to support this contention comes from previous studies in which the amniotic fluid concentrations of tumor necrosis factor and interleukin-1alpha, interleukin-1beta, and IL-6 were significantly higher in patients with preterm labor and intact membranes than in patients with preterm premature rupture of membranes (both with microbial invasion of the amniotic cavity) [16,17,28,29,30,31].

A major finding of this study is that amniotic fluid IL-6 concentration was an independent explanatory variable of admission-to-delivery interval and the occurrence of neonatal complications. Moreover, amniotic fluid IL-6 concentrations added significantly to gestational age when modeling the occurrence of neonatal morbidity and mortality. These observations indicate that measurements of amniotic fluid IL-6 would add new and significant information to the clinician.

A practical consideration is whether assays for IL-6 are clinically available and practical. Several biotechnology companies offer immunoassays for the determination of IL-6 in tissue culture media and biologic fluids. IL-6 is a group of differentially phosphorylated glycoproteins. It is extremely important that assays are carefully and extensively validated for the biologic fluid of interest. Insofar as the speed of the assay, immunoassays can be configured to produce very quick results. It is possible to develop a dip-stick test for immediate results in the antepartum and labor and delivery ward. These formats shall narrow the distance between research and clinical application.

In this study we have analyzed the performance of other rapid tests (white blood cell count, amniotic fluid glucose, and Gram stain) for the diagnosis of microbial invasion. Institutions lacking the resources to conduct IL-6 determinations may choose to perform a combination of other rapid and inexpensive tests. However, it is clear that these tests do not perform as well as IL-6 in the prediction of interval to delivery and neonatal complications. Further prospective studies are required to determine if the cutoff values identified in this study for the different amniotic fluid tests are optimal.

In summary, we have demonstrated that amniotic fluid IL-6 determinations are of clinical value in the detection of microbial invasion of the amniotic cavity and in the identification of the patients at risk for impending preterm delivery and neonatal complications.

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A complete set of tables is available from the authors on request.

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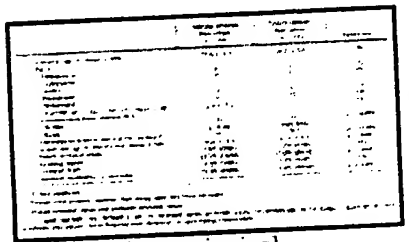

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Table I. Clinical characteristics of patients with preterm premature rupture of membranes according to amniotic fluid culture results

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Mechanisms of Disease: Premature Rupture of the Fetal Membranes

[Review Articles]

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Outline

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Graphics

- Figure 1
- Figure 2

The membranes surrounding the amniotic cavity are composed of the amnion and the chorion, which are closely adherent layers consisting of several cell types, including epithelial cells, mesenchymal cells, and trophoblast cells, embedded in a collagenous matrix. They retain amniotic fluid, secrete substances both into the amniotic fluid and toward the uterus, and guard the fetus against infection ascending the reproductive tract. The membranes normally rupture during labor. Premature rupture of the fetal membranes is defined as rupture of the membranes before the onset of labor. [1] Premature rupture of the membranes occurring before 37 weeks' gestation is usually referred to as preterm premature rupture of the membranes. Despite advances in perinatal care, premature rupture of the membranes and preterm premature rupture of the membranes continue to be important obstetrical complications. At term, 8 to 10 percent of pregnant women present with premature rupture of the membranes; these women are at increased risk for intrauterine infection when the interval between the membrane rupture and delivery is prolonged. [1] Preterm premature rupture of the membranes occurs in approximately 1 percent of all pregnancies and is associated with 30 to 40 percent of preterm deliveries. It is thus the leading identifiable cause of preterm delivery (after less than 37 completed weeks' gestation) and its complications, including respiratory distress syndrome, neonatal infection, and intraventricular hemorrhage.

Obstetricians have traditionally attributed rupture of the membranes to physical stress, particularly that associated with labor. However, more recent evidence suggests that membrane rupture is also related to biochemical processes, including disruption of collagen within the extracellular matrix of the amnion and the chorion and programmed death of cells in the fetal membranes. It has been proposed that the fetal membranes and the maternal uterine lining (decidua) respond to various stimuli, including membrane stretching and infection of the reproductive tract, by producing mediators, such as prostaglandins, cytokines, and protein hormones, that govern the activities of matrix-degrading enzymes. We review here the association between the degradation of the extracellular matrix within the fetal membranes and premature rupture of the membranes, in an effort to understand better the pathophysiology of such ruptures and identify potentially effective interventions.

Structure of the Fetal Membranes

The human amnion is composed of five distinct layers (Figure 1). [2] It contains no blood vessels or nerves; the nutrients it requires are supplied by

the amniotic fluid. The innermost layer, nearest the fetus, is the amniotic epithelium. Amniotic epithelial cells secrete collagen types III and IV and noncollagenous glycoproteins (laminin, nidogen, and fibronectin) that form the basement membrane, the next layer of the amnion.

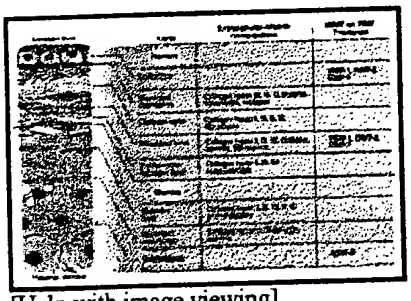

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Figure 1. Schematic Representation of the Structure of the Fetal Membranes at Term. The extracellular-matrix composition of each layer and the production sites of matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP) are shown.

The compact layer of connective tissue adjacent to the basement membrane forms the main fibrous skeleton of the amnion. The collagens of the compact layer are secreted by mesenchymal cells in the fibroblast layer. [3] Interstitial collagens (types I and III) predominate and form parallel bundles that maintain the mechanical integrity of the amnion. [4] Collagen types V and VI form filamentous connections between the interstitial collagens and the epithelial basement membrane. [4] There is no interposition of amorphous ground substance between collagen fibrils in amniotic connective tissue at term, so the amnion maintains its tensile strength throughout the late stages of normal pregnancy.

The fibroblast layer is the thickest of the amniotic layers, consisting of mesenchymal cells and macrophages within an extracellular matrix. [4] The collagens in this layer form a loose network with islands of noncollagenous glycoproteins.

The intermediate layer (spongy layer, or zona spongiosa) lies between the amnion and the chorion. Its abundant content of hydrated proteoglycans and glycoproteins gives this layer its "spongy" appearance in histologic preparations, and it contains a nonfibrillar meshwork of mostly type III collagen. The intermediate layer absorbs physical stresses by permitting the amnion to slide on the underlying chorion, which is firmly adherent to the maternal decidua.

Although the chorion is thicker than the amnion, the amnion has greater tensile strength. The chorion resembles a typical epithelial membrane, with its polarity directed toward the maternal decidua. As pregnancy progresses, trophoblastic villi within the chorionic layer of the reflected fetal membranes (free of the placenta) regress. Beneath the cytotrophoblast layer (closer to the fetus) are the basement membrane and the chorionic connective tissue, which is rich in collagen fibrils.

The fetal membranes display regional variation that distinguishes the membranes overlying the placenta from the reflected membranes. Although there is no evidence of preset weak points where the membranes break, care must be taken to avoid overlooking localized changes in the membrane structure and composition in studies of premature rupture of the membranes.

Natural History and Management of Fetal-Membrane Rupture

After premature rupture of the membranes at term, 70 percent of women begin to labor within 24 hours, and 95 percent within 72 hours. [5] After preterm premature rupture of the membranes, the latency period from membrane rupture to delivery decreases inversely with advancing gestational age. For example, at 20 to 26 weeks' gestation, the mean latency period is 12 days; at 32 to 34 weeks' gestation, it is only 4 days. [6]

Given the natural history of the relatively rapid progression to labor after premature rupture of the membranes at term, the goal of management is to minimize the risk of intrauterine infection without increasing the incidence of cesarean delivery. In published series, the rate of neonatal sepsis after preterm premature rupture of the membranes ranges from 2 to 20 percent, and the incidence of neonatal death caused by infection is approximately 5 percent. When the fetal membranes rupture at term or before, the options are expectant management (with close observation for signs of labor, nonreassuring fetal-heart-rate patterns, or intrauterine infection) or induction of labor.

Mechanisms of Fetal-Membrane Rupture Preceding and During Labor

Intrapartum rupture of the fetal membranes has been attributed to generalized weakening due to uterine contractions and repeated stretching. The tensile strength of the membranes is reduced in specimens obtained after labor as compared with those obtained during cesarean delivery without labor. [7] Generalized weakness of the membranes has been more difficult to establish when prematurely ruptured membranes have been compared with membranes that were artificially ruptured during labor. [8] Membranes that rupture prematurely, however, appear to be focally defective rather than generally weakened. The area near the rupture site has been described as a "restricted zone of extreme altered morphology" that is characterized by marked swelling and disruption of the fibrillar collagen network within the compact, fibroblast, and spongy layers. [9] Because this zone does not include the entire rupture site, it may appear before membrane rupture and represent the initial breakpoint.

Despite the divergent characteristics of premature rupture of the membranes and intrapartum rupture of the membranes, there is little evidence to suggest that the mechanisms that predispose women to the former are not identical to those that normally precede labor. This has led

to the view that premature rupture of the membranes represents an acceleration or exaggeration of the processes precipitating spontaneous rupture of the membranes during labor. Consequently, investigators have frequently combined instances of preterm premature rupture of the membranes, premature rupture of the membranes at term, and rupture of the membranes during labor when describing mechanisms of membrane rupture. This practice, however, may obscure important differences among these events.

Changes in Collagen Content, Structure, and Catabolism

The maintenance of the tensile strength of fetal membranes appears to involve an equilibrium between the synthesis and the degradation of the components of the extracellular matrix. It has been proposed that changes in the membranes, including decreased collagen content, altered collagen structure, and increased collagenolytic activity, are associated with premature rupture of the membranes.

Connective-Tissue Disorders and Nutritional Deficiencies as Risk Factors

Although there are conflicting data regarding changes in the composition of fetal-membrane collagen in association with the length of gestation and membrane rupture, a decline in membrane collagen content or a change in collagen structure probably precedes rupture of the membranes. [10-12]

Connective-tissue disorders are associated with weakened fetal membranes and an increased incidence of preterm premature rupture of the membranes. Ehlers-Danlos syndrome, a group of at least 11 heritable disorders of connective tissue characterized by hyperelasticity of the skin and joints, is caused by various defects in the synthesis or structure of collagen. Among 18 patients with Ehlers-Danlos syndrome whose birth histories were available, 13 (72 percent) were delivered prematurely after preterm premature rupture of the membranes, as compared with 1 of 16 unaffected siblings, and this one instance occurred in a twin gestation in which the other twin had Ehlers-Danlos syndrome. [13] Thus, pregnancies in which the fetus is affected with Ehlers-Danlos syndrome are dramatic examples of preterm premature rupture of the membranes associated with abnormal collagen content and structure.

Nutritional deficiencies that predispose women to abnormal collagen structure have also been associated with an increased risk of preterm premature rupture of the membranes. Collagen cross-links, which are formed in a series of reactions initiated by lysyl oxidase, increase the tensile strength of fibrillar collagens. Lysyl oxidase is produced by amniotic mesenchymal cells, which lay down the collagenous compact layer of the amnion. [14] Lysyl oxidase is a copper-dependent enzyme, and women with premature rupture of the membranes have lower copper concentrations in maternal and umbilical-cord serum than women whose fetal membranes are artificially ruptured during labor. [15] Similarly, women with low serum

concentrations of ascorbic acid, which is required for the formation of the triple helical structure of collagen, have a higher rate of premature rupture of the membranes than those with normal serum concentrations. [16] Tobacco smoking, which independently increases the risk of preterm premature rupture of the membranes, has been associated with decreased serum concentrations of ascorbic acid. [17] In addition, the cadmium in tobacco has been found to increase the metal-binding protein metallothionein in trophoblasts, which may result in sequestration of copper. These data suggest that the decreased availability of copper and ascorbic acid may contribute to an abnormal structure of fetal-membrane collagen in smokers. Collectively, reduced collagen cross-linking (possibly due to dietary deficiencies or behavioral activities) may predispose women to premature membrane rupture.

Increased Collagen Degradation

The degradation of collagen is mediated primarily by matrix metalloproteinases, which are inhibited by specific tissue inhibitors and other protease inhibitors. The matrix metalloproteinases are a family of enzymes produced by various types of cells that hydrolyze at least one component of the extracellular matrix. Because of the various substrate specificities of matrix metalloproteinases, effective catabolism of the many component molecules in the extracellular matrix requires the concerted actions of several enzymes. The interstitial collagenases matrix metalloproteinase-1 (MMP-1) and MMP-8 cleave the triple helix of the fibrillar collagens (types I and III), which are then further degraded by the gelatinases MMP-2 and MMP-9. These gelatinases also cleave type IV collagen, fibronectin, and proteoglycans. In human fetal membranes, MMP-1 and MMP-9 messenger RNA and protein have been colocalized to amniotic epithelial cells and chorionic trophoblasts. [18,19] Thus, the compact (collagenous) layer of the fetal membranes is sandwiched between two layers of cells that produce matrix metalloproteinases.

Tissue inhibitors of metalloproteinases form 1:1 stoichiometric complexes with matrix metalloproteinases and inhibit their proteolytic activity. Tissue inhibitor of metalloproteinase-1 (TIMP-1) binds to activated MMP-1, MMP-8, and MMP-9, and TIMP-2 binds to latent and active forms of MMP-2. The more recently described TIMP-3 and TIMP-4 appear to inhibit matrix metalloproteinases as efficiently as TIMP-1. Coordinated activities of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases are essential to the process of extracellular-matrix remodeling.

The integrity of the fetal membranes remains unaltered throughout most of gestation, perhaps in part because of a combination of low matrix-metalloproteinase activity and a relatively higher concentration of TIMP-1. [20] Near the time of delivery, however, the balance between activated matrix metalloproteinases and their tissue inhibitors shifts toward

proteolytic degradation of the extracellular matrix of the fetal membranes. In the amnion of rats, activities of interstitial collagenase and MMP-9 increase before the onset of active labor. [21,22] In human amnion and chorion, MMP-9 activity increases and TIMP-1 concentrations decrease dramatically with labor. [18,20] Analyses of membranes collected from women at the time of cesarean delivery (with and without labor) and after spontaneous labor and delivery suggest that MMP-1 activity increases before labor, MMP-9 and MMP-3 activities increase during labor, and TIMP-1 concentrations increase after delivery. [23] These changes may reflect a coordinated progression of events preceding and during parturition, resulting in the controlled degradation of collagen within the fetal membranes.

Premature rupture of the membranes may also be caused by an imbalance between the activities of matrix metalloproteinases and their tissue inhibitors, leading to inappropriate degradation of the membranes' extracellular matrixes. Collagenase activity is increased in prematurely ruptured membranes at term. [12] Overall, protease activity is increased in membranes of women with preterm premature rupture of the membranes, the predominant activity being that of MMP-9. [24] Furthermore, gelatinolytic activity corresponding to latent and active forms of MMP-9 is increased and the concentration of TIMP-1 is low in amniotic fluid obtained from women whose pregnancies were complicated by preterm premature rupture of the membranes. [20] However, because specimens in these studies were obtained after membrane rupture, we cannot conclude with certainty that collagen degradation in the fetal membranes precedes membrane rupture.

Other observations suggest that physiologic and pathologic degradation of the extracellular matrix is associated with labor and delivery. Interstitial-collagenase activity increases dramatically in cervical tissue during cervical dilation in human parturition. [25] Periodontal disease, in which there is increased matrix-metalloproteinase activity in gingival tissues, has been reported to be an independent risk factor for preterm delivery. [26] This finding raises the interesting possibility that some women have a genetic predisposition to extracellular-matrix degradation due to increased matrix-metalloproteinase activity that may be manifested clinically as periodontitis, premature cervical dilatation, or premature rupture of the membranes.

Clinical Factors Associated with Collagen Degradation and Premature Rupture of the Membranes

Infection

Obstetricians have long debated whether intrauterine infection is a cause or a consequence of premature rupture of the fetal membranes. There is indirect evidence that genital tract infection precipitates rupture of the membranes in animals and humans. In pregnant rabbits, cervical inoculation with Escherichia coli resulted in positive cultures for E. coli in the amniotic

fluid and decidual tissue of 97 percent of the treated animals and preterm delivery in half the treated animals. In contrast, cervical inoculation with saline resulted in no infections or preterm births. [27] The identification of pathologic microorganisms in human vaginal flora soon after membrane rupture provides support for the concept that bacterial infection may have a role in the pathogenesis of premature membrane rupture. [28] Epidemiologic data demonstrate an association between colonization of the genital tract by group B streptococci, Chlamydia trachomatis, Neisseria gonorrhoeae, and the microorganisms that cause bacterial vaginosis (vaginal anaerobes, Gardnerella vaginalis, Mobiluncus species, and genital mycoplasmas) and an increased risk of preterm premature rupture of the membranes. [29-32] Furthermore, in some studies treatment of infected women with antibiotics decreased the rate of preterm premature rupture of the membranes. [32,33]

Intrauterine infection may predispose women to rupture of the fetal membranes through any of several mechanisms, each of which induces degradation of the extracellular matrix. Several organisms that are commonly present in the vaginal flora, including group B streptococci, Staphylococcus aureus, Trichomonas vaginalis, and the microorganisms that cause bacterial vaginosis, secrete proteases that can degrade collagen and weaken the fetal membranes. [34,35] In an in vitro system, proteolysis of the fetal membrane matrix can be inhibited by the addition of an antibiotic. [33]

The host inflammatory response to bacterial infection constitutes another potential mechanism that may partly account for the association between bacterial infection of the genital tract and premature rupture of the membranes. The inflammatory response is mediated by polymorphonuclear neutrophils and macrophages that are recruited to the site of infection and produce cytokines, matrix metalloproteinases, and prostaglandins. Inflammatory cytokines, including interleukin-1 and tumor necrosis factor (alpha), are produced by stimulated monocytes, and these cytokines increase MMP-1 and MMP-3 expression at the transcriptional and posttranslational levels in human chorionic cells. [36,37]

Bacterial infection and the host inflammatory response also induce prostaglandin production by the fetal membranes, which is thought to increase the risk of preterm premature rupture of the membranes by causing uterine irritability and collagen degradation within the membranes. Certain strains of vaginal bacteria produce phospholipase $A(_2)$), which releases the prostaglandin precursor arachidonic acid from membrane phospholipids within the amnion. Furthermore, the immune response to bacterial infection includes the production of cytokines by activated monocytes that increase prostaglandin $E(_2)$) production by chorionic cells. [37] Cytokine stimulation of prostaglandin $E(_2)$) production by the amnion and chorion appears to involve induction of cyclooxygenase II, the enzyme that converts arachidonic acid into prostaglandins. [38] The precise regulation of

prostaglandin E_2)) synthesis in relation to bacterial infection and the host inflammatory response is not understood, and a direct link between prostaglandin production and premature rupture of the membranes has not been established. However, prostaglandins (specifically prostaglandin E_2)) and prostaglandin F_2)($_{alpha}$))) are considered to be mediators of labor in all mammals, and prostaglandin E_2)) diminishes collagen synthesis in fetal membranes and increases MMP-1 and MMP-3 expression in human fibroblasts. [39,40]

Another component of the host response to infection is the production of glucocorticoids. In most tissues the antiinflammatory action of glucocorticoids is mediated by suppression of prostaglandin production. However, in some tissues, including the amnion, glucocorticoids paradoxically stimulate prostaglandin production. Furthermore, dexamethasone reduces the synthesis of fibronectin and type III collagen in primary cultures of amniotic epithelial cells. [41] These findings suggest that glucocorticoids produced in response to the stress of microbial infection facilitate rupture of the fetal membranes.

Despite these findings, there has been no conclusive demonstration that infection precedes premature rupture of the fetal membranes in humans. Nonetheless, microbial infection and the host inflammatory response may at the very least increase the activity of matrix metalloproteinases in the fetal membranes and be involved in the pathogenesis of some membrane ruptures.

Hormones

Progesterone and estradiol suppress extracellular-matrix remodeling in reproductive tissues. Both hormones decrease concentrations of MMP-1 and MMP-3 and increase the concentrations of tissue inhibitors of metalloproteinases in the cervical fibroblasts of rabbits. [42] High concentrations of progesterone decrease the production of collagenase in the cervical fibroblasts of guinea pigs, although lower concentrations of progesterone and estradiol stimulate the production of collagenase in pregnant guinea pigs. [43] Relaxin, a protein hormone that regulates the remodeling of connective tissues, is produced locally in the decidua and placenta and reverses the inhibitory effects of estradiol and progesterone by increasing MMP-3 and MMP-9 activities in fetal membranes. [44] Expression of the relaxin gene is increased before labor in human fetal membranes at term. [23] Although it is important to consider the roles of estrogen, progesterone, and relaxin in reproductive processes, their involvement in the process of fetal-membrane rupture remains to be defined.

Programmed Cell Death

Programmed cell death, or apoptosis, has been implicated in the remodeling of various reproductive tissues, including those of the uterus and cervix. Apoptosis is characterized by the nuclear DNA fragmentation and catabolism of 28S ribosomal RNA subunits that are required for protein synthesis. In rats (which have a 21-day gestation), amniotic epithelial cells undergo apoptotic cell death as labor approaches. [22] This cell death appears to follow the start of extracellular-matrix degradation, suggesting that it is a consequence and not a cause of catabolism of the extracellular matrix of the amnion.

Human amnion and chorion obtained at term after premature rupture of the membranes contain many apoptotic cells in areas adjacent to the rupture site and fewer apoptotic cells in other areas of the membranes. [45] Furthermore, in cases of chorioamnionitis, apoptotic amniotic epithelial cells are seen in conjunction with adhesive granulocytes, suggesting that the host immune response may accelerate cell death in fetal membranes. [45] Although apoptotic changes have been identified in fetal membranes immediately before delivery, the mechanisms regulating apoptosis and the subsequent effects on the tensile strength of fetal membranes have yet to be elucidated.

Membrane Stretch and Premature Rupture of the Membranes

Uterine overdistention due to both polyhydramnios and multifetal gestation induces membrane stretch and increases the risk of premature rupture of the membranes. Mechanical stretching of the fetal membranes up-regulates the production of several amniotic factors, including prostaglandin $E(_2)$) and interleukin-8. Stretch also increases MMP-1 activity within the membranes. [46] As stated above, prostaglandin $E(_2)$) increases uterine irritability, decreases synthesis of fetal-membrane collagen, and increases production of MMP-1 and MMP-3 by human fibroblasts. [39,40] Interleukin-8, which is produced by amniotic and chorionic cells, is chemotactic for neutrophils and stimulates collagenase activity. The production of interleukin-8, which is present in low concentrations in the amniotic fluid during the second trimester but in much higher concentrations late in gestation, is inhibited by progesterone. Thus, amniotic production of interleukin-8 and prostaglandin $E(_2)$) represents biochemical changes in the fetal membranes that may be initiated by physical forces (membrane stretch), reconciling the hypothesis of force-induced and biochemically induced membrane rupture.

Predicting Preterm Premature Rupture of the Membranes

Markers of degradation of the extracellular matrix of fetal membranes could be used to identify women who are at risk for premature rupture of the membranes and preterm delivery. The most extensively studied candidate marker is fetal fibronectin, which is present in the extracellular matrix of fetal membranes and is structurally different from the fibronectin of adult tissues. The production of fetal fibronectin by human amniotic cells is

stimulated by inflammatory mediators (including interleukin-1 and tumor necrosis factor (alpha)) that are considered important in initiating preterm labor. [47]

In the second and third trimesters of pregnancy, the presence of fetal fibronectin in cervicovaginal secretions probably reflects degradation of the extracellular matrix at the interface of the chorionic and decidual layers. Measurements of fetal fibronectin in these secretions have been used to identify a subgroup of women at high risk for preterm delivery. [48] Fetal fibronectin is most sensitive for predicting preterm birth at less than 28 weeks' gestation (sensitivity, 63 percent); however, the positive predictive value for preterm birth is less than 33 percent at all gestational ages, and there is no evidence that this test can be used to predict preterm premature rupture of the membranes and reduce the rate of preterm birth. [49,50] Tests based on the detection of other molecules, such as specific matrix metalloproteinases, have not yet been applied to clinical practice.

Prevention of Preterm Premature Rupture of the Membranes

There has been considerable interest in the development of general and specific inhibitors of matrix metalloproteinases for the treatment of periodontal disease and arthritis and for the prevention of tumor metastasis. These agents include tetracycline antibiotics, synthetic matrix-metalloproteinase inhibitors such as batimastat (which selectively chelates the zinc atom at the active site of the enzymes), and the native inhibitors TIMP-1 and TIMP-2. The ability of such substances to prevent or retard changes in the extracellular matrix of fetal membranes before preterm premature rupture occurs has yet to be evaluated.

Conclusions

The cause of premature rupture of the fetal membranes is almost certainly multifactorial (Figure 2). Traditionally, rupture of the fetal membranes has been attributed to increasing physical stresses that weaken the membranes. At the molecular level, premature rupture of the membranes appears to result from diminished collagen synthesis, altered collagen structure, and accelerated collagen degradation, possibly in association with concurrent cellular changes within the fetal membranes. These hypotheses are not mutually exclusive, and biophysical stresses may amplify these biochemical changes.

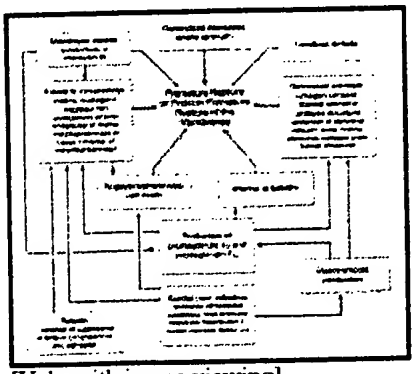

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Figure 2. Schematic Diagram of the Various Mechanisms That Have Been Proposed to Result in Premature Rupture or Preterm Premature Rupture of the Fetal Membranes.

Present research priorities include elucidation of the normal biologic processes of the fetal membranes, including extracellular-matrix remodeling, programmed cell death, and the response to membrane stretch as pregnancy progresses. We need to learn how exogenous risk factors, including nutritional deficiencies, smoking, and infection, promote premature rupture of the membranes. A more thorough understanding of extracellular-matrix degradation in the amnion and the chorion may allow us to reduce the incidence of preterm delivery due to preterm premature rupture of the membranes, possibly with agents that delay matrix degradation.

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Cas -control study of antenatal and intrapartum risk factors for cerebral palsy in very preterm singleton babies

[Articles]

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Outline

Graphics

Summary

The increase in survival of very preterm babies during the 1980s was accompanied by a sharp increase in the rate of cerebral palsy in this group. The relation between antenatal and intrapartum factors and cerebral palsy in such babies has not been well defined. To identify

adverse and protective antenatal and intrapartum factors we undertook a case-control study of 59 very preterm babies who developed cerebral palsy, identified from a population-based register, and 234 randomly selected controls.

The frequency of cerebral palsy decreased with increasing gestational age and birthweight.Antenatal complications occurred in 215 (73 percent) of the women with preterm deliveries. Factors associated with an increased risk of cerebral palsy after adjustment for gestational age were chorioamnionitis (odds ratio 4.2 [95 percent CI 1.4-12.0]) prolonged rupture of membranes (2.3 [1.2-4.2]), and maternal infection (2.3 [1.2-4.5]). Pre-eclampsia was associated with a reduced risk of cerebral palsy (0.4 [0.2-0.9]), as was delivery without labour (0.3 [0.2-0.7]). There was no increased risk of cerebral palsy with intrauterine growth retardation (1.0 [0.9-1.1]).

The effect of rigorous management of adverse antenatal factors on the frequency of cerebral palsy in very preterm babies should be tested in randomised controlled trials.

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Introduction

During the early 1980s, there was an increase in the proportion of very preterm babies who survived, owing to advances in neonatal intensive care. This increase was accompanied by a sharp rise in the rate of cerebral palsy among babies weighing less than 1500 g at birth. [1]

There are several possible explanations for this finding. It may reflect the survival of babies who have sustained an in-utero ischaemic insult that resulted in both preterm birth and white matter damage; [1] this damage may be manifest later as cerebral palsy. Alternatively, it could be due to the survival of a greater proportion of immature babies who are particularly vulnerable to haemorrhage and ischaemia in the intrapartum and neonatal period. [2] These babies would not have survived before the advances in neonatal intensive care. A third possibility is that cerebral palsy represents the endpoint of a continuum of adverse effects that occur throughout the period when the brain is especially vulnerable to ischaemia. These events may occur before, during, or after birth. A better understanding of the aetiology of preterm cerebral palsy is necessary for both preventive strategies and treatments to be developed.

Clinical events that might be antecedents of cerebral palsy have been sought in several different ways. Prospective cohort studies of preterm babies have identified the clinical associations of ultrasound changes which are detected postnatally and which are predictive of later cerebral palsy. [3-5] These studies have focused mainly on intrapartum and neonatal factors, with less information on possible antenatal antecedents of cerebral palsy. Associations between cerebral palsy and antenatal or intrapartum factors have generally been sought through case-control studies. O'Shea and colleagues' study of very-low-birthweight babies [6] showed an increased risk of cerebral palsy with chorioamnionitis and antepartum haemorrhage and a decreased risk with delivery without labour. These findings contrasted

with those from similar studies in term babies, in which intrauterine growth retardation and pre-eclampsia were identified as risk factors for cerebral palsy. [7] In earlier studies, which included babies of all gestational ages, different associations with cerebral palsy were found. For example, Nelson and Ellenberg [8] found that maternal mental retardation, low birthweight, fetal malformation, and breech presentation were associated with subsequent cerebral palsy, whereas Torfs et al [9] found associations between birth defects and low placental weight and cerebral palsy. It seems likely therefore that the risk factors for cerebral palsy differ between preterm and mature babies.

Pre-eclampsia is associated with a reduced risk of subependymal and intraventricular haemorrhage in very-low-birthweight babies. [10,11] Nelson and Grether [12] found a reduced risk of cerebral palsy in such babies whose mothers were given magnesium sulphate for pre-eclampsia or to prevent preterm labour. This observation raises questions about the role of pre-eclampsia in the aetiology of cerebral palsy in preterm babies which, together with the uncertainty about risk factors for cerebral palsy in this group, led to our study.

We used a population register of cerebral palsy to identify antenatal and intrapartum antecedents of cerebral palsy in a group of children born as singletons before 32 weeks of gestation.

Subjects and methods

Selection of subjects

The selection criteria were singleton birth at less than 32 completed weeks of gestation; mother resident in Oxford district or West Berkshire; birth between 1984 and 1990; and survival to hospital discharge. Multiple births were excluded from this study since there is evidence that the risk factors for cerebral palsy in that group differ from those in singleton infants. [13]

Gestational age was estimated by means of menstrual dates and an ultrasound scan done before 20 weeks' gestation. The scan date was preferred if the menstrual date was uncertain or there was a discrepancy of more than 14 days between the two estimates.

Cases - 59 children with cerebral palsy were identified from the Oxford region register of early childhood impairments. [14] The definition of cerebral palsy used by the register is permanent impairment of voluntary movement or posture presumed to be due to permanent damage to the immature brain. The register includes children of women who were resident within the former Oxford health region when the child was born. Many sources of ascertainment are used to compile the register, and the condition of the children is assessed at the ages of 3 and 5 years.

Controls - There were 474 eligible babies who survived to discharge and did not develop cerebral palsy. These babies were identified from two

sources to ensure maximum ascertainment - the hospital admission registers and birth tapes from the Oxford Record Linkage Study.

Based on an audit of preterm birth at the John Radcliffe Hospital (unpublished), we assumed that an adverse antenatal factor would be present in 25-30 percent of controls. We predicted that a study population of 59 cases of cerebral palsy with four controls for each case would be large enough to detect for each obstetric factor an odds ratio of 2.5 with 80 percent power and alpha of 0.05. We wished therefore, to select half of the potential controls. A coin was tossed for each available subject and 235 controls (about four per case) were randomly selected.

The approval of the Oxfordshire and West Berkshire ethics committees was obtained before the start of the study.

Data sources

The obstetric notes of mothers included in the study were reviewed by a researcher unaware of the children's outcome. A detailed data-set was completed; 130 variables included demographic data, maternal medical history, family history, previous obstetric history, and history of the relevant pregnancy.

Pre-eclampsia (diastolic pressure greater than or equal to 90 mm Hg at least twice, plus proteinuria), antepartum haemorrhage (after 20 weeks' gestation), prolonged rupture of the membranes (greater than 24 h), chorioamnionitis (infection or inflammation of the membranes diagnosed by at least two of clinical, microbiological, and histological evidence), and maternal infection (urinary tract infection or pyelonephritis confirmed on midstream urine; temperature greater than 38.5 degrees C for greater than 24 h; any other microbiologically confirmed infection than chorioamnionitis) were of especial interest. Details of diagnosis, onset, duration, and management were recorded.

Many of the babies had had detailed antenatal monitoring including ultrasound scanning, cardiotocography, and doppler studies. Ultrasound scan reports were reviewed for evidence of fetal anomalies, intrauterine growth retardation (weight greater than 2 SD below the mean on John Radcliffe Hospital percentile charts estimated by serial measurements of abdominal circumference), abnormal liquor volume (vertical liquor pool less than 2.0 cm [oligohydramnios] or greater than 8.0 cm [polyhydramnios] on ultrasound assessment closest to delivery), or poor biophysical profile. Intrapartum and latest antenatal cardiotocographs were analysed independently by two investigators (DJM, SS).

The terms used to describe and classify the intrapartum cardiotocographs were those used in the Dublin trial of continuous monitoring of fetal heart rate. [15] Antenatal cardiotocographs were computerised in many cases

with a set of defined features summarised as normal, suspicious, or abnormal (Oxford sonicaid 8000). A system modified from Cheng et al [16] was used to analyse the non-computerised cardiotocographs to assign a scoring system comparable with the computerised system.

When the two observers disagreed, a third person was asked to arbitrate. This was rarely necessary because the criteria were precise. The observers in all these reviews were unaware of outcome.

Statistical methods

The odds ratio associated with a given factor estimates the risk of cerebral palsy when the factor is present relative to that when the factor is absent. Odds ratios adjusted for gestational age were calculated by logistic regression. Adjustment for additional confounding variables was also made by logistic regression, where numbers were adequate. Comparisons of continuous data were made with Student's t test. Trends in the rates of survival and cerebral palsy among survivors by gestational age and birthweight were tested by chi square tests for trend.

Results

Of 638 singleton babies born alive at less than 32 completed weeks' gestation to mothers resident in Oxford district and West Berkshire during 1984-90, 105 died before discharge. The survival rate increased with gestational age and with birthweight. The rate of cerebral palsy among survivors decreased with increasing gestational age and with increasing birthweight Figure 1.

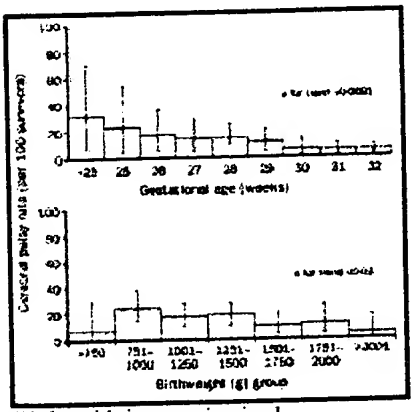

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Figure 1. Rate of cerebral palsy according to gestational age and birthweight

Obstetric notes were available for the mothers of all 59 children with cerebral palsy and the mothers of 230 control children; neonatal notes provided the data for a further four controls. Neither obstetric nor neonatal notes could be found for one of the controls selected. Analyses were

therefore based on 59 cases of cerebral palsy and 234 controls.

Mean gestational age at birth was 1.3 (95 percent CI 0.7-1.9) weeks less for the cerebral palsy cases than for the controls (mean 28.6 [SD 2.3; range 24-32] vs 29.9 [1.9; 23-32] weeks; p less than 0.0001). Since this difference confounds any comparison between cases and controls, odds ratio estimates were adjusted for gestational age. Birthweight was also significantly lower in cases than in controls (1282 [343] vs 1426 [413] g; difference=144 [29-259] g; p less than 0.0001). Further adjustment for birthweight did not affect the results, so odds ratios are reported without this adjustment.

Maternal factors

High parity was associated with an increased risk of cerebral palsy Table 1. No other demographic factor analysed was a significant risk factor. Factors previously associated with preterm birth (poor obstetric history, unmarried status, low maternal age or body weight) occurred at similar rates in cases and controls.

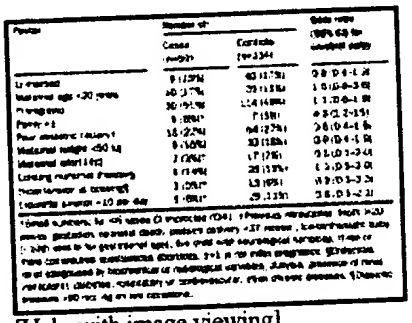

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Table 1. Maternal factors among cases of cerebral palsy and controls

Infertility and induced conception were not risk factors for cerebral palsy but an effect might have been missed because multiple births were excluded from the study. There were no significant differences in the medical histories of the two groups of women or in their smoking habits.

Antenatal factors

Antenatal complications were very common in these pregnancies; 215 (73 percent) women had at least one complication Table 2. Complications associated with cerebral palsy after adjustment for gestational age were prolonged rupture of the membranes, chorioamnionitis, and other maternal infection. The association with chorioamnionitis persisted after adjustment for the confounding effects of gestational age, type of delivery, and other maternal infection (odds ratio 3.9 [1.3-11]). Placental abruption was associated with an increased risk of cerebral palsy on univariate analysis (2.1 [1.1-4.2]) but most cases occurred earlier in gestation and the association was no longer significant when adjustment was made for

gestational age.

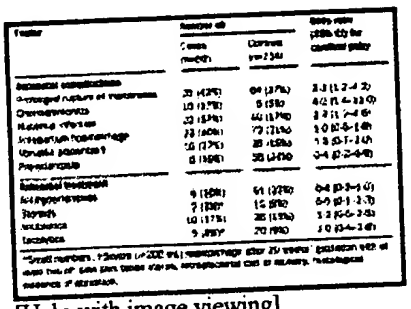

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Table 2. Antenatal variables among cases of cerebral palsy and controls

Pre-eclampsia was associated with a significantly reduced risk of cerebral palsy. The independent effects of pre-eclampsia and delivery without labour could not be established by logistic regression since none of these babies was delivered after spontaneous labour.

There was a very low rate of administration of corticosteroids and tocolytics in the study groups with no significant differences between the cases and controls. Of 260 women who had a serum alphafetoprotein assay, 11 (4 percent) had results two or more multiples above the median but there was no difference between cases and controls Table 3. This percentage is, however, double that expected for high alphafetoprotein results. The usual rate in this region is 2 percent for deliveries at all gestations.

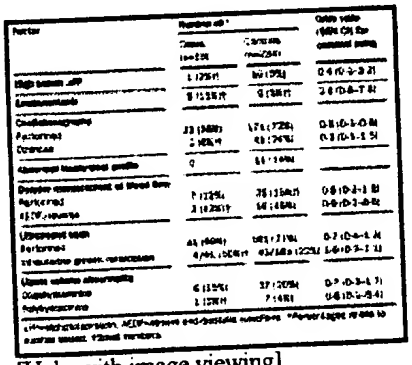

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Table 3. Antenatal investigations and monitoring among cases of cerebral palsy and controls

There were no significant differences between cases and controls in terms of ominous antenatal cardiotocography, low biophysical scores, reverse or absent end-diastolic velocity waveforms, and abnormalities of liquor volume.

Intrauterine growth retardation was diagnosed on ultrasound scan in 44 of 222 (20 percent) women scanned and was not associated with an increased

risk of cerebral palsy. Of these babies, 35 (80 percent) were born to mothers with pre-eclampsia.

Intrapartum factors

Delivery without labour was associated with a decreased risk of cerebral palsy Table 4. This association was independent of gestational age and antenatal complications (0.4 [0.2-0.8]). Caesarean section in labour was associated with an increased risk of cerebral palsy. Vaginal delivery, both by the vertex and by the breech, was associated with an increased risk of cerebral palsy but these odds ratios did not reach statistical significance. There were no significant differences in the numbers of ominous CTGs between cases and controls for first or second stage of labour.

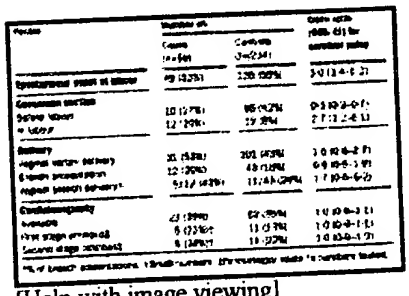

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Table 4. Intrapatum factors among cases of cerebral palsy and controls

Characteristics of babies and condition at birth

There were 170 (58 percent) boys and 123 (42 percent) girls in the study group Table 5. The distribution of the sexes was similar among cases and controls. At birth, 34 (12 percent) babies were small for gestational age compared with 44 babies reported as growth-retarded on ultrasound scan; 32 of the small-for-gestational-age babies had been identified antenatally. There was no significant increase in risk of cerebral palsy in small-for-gestational-age babies on univariate analysis or after adjustment for the confounding effects of gestational age, delivery without labour, and pre-eclampsia (1.1 [0.3-4.2]).

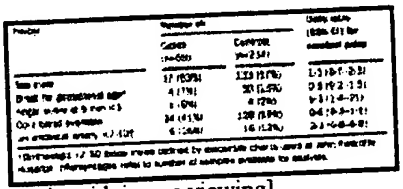

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Table 5. Characteristics of babies at birth

An Apgar score of 3 or less at 5 min was significantly associated with an increased risk of cerebral palsy Table 5. An umbilical artery pH of 7.10 or less was more frequent among babies with cerebral palsy than among

controls but the difference was no greater than would be expected by chance.

Discussion

In this study of preterm births, gestational age had the strongest association with cerebral palsy. Because an increasing proportion of babies of very low gestational age are surviving, long-term outcome of this high-risk group needs to be monitored.

52 antenatal factors were investigated; prolonged rupture of the membranes, maternal infection, and chorioamnionitis were associated with a significantly increased risk of cerebral palsy after adjustment for the confounding effect of gestational age. Previous studies have found chorioamnionitis in association with preterm delivery in up to 50 percent of very-low-birthweight infants. [17-19] Nelson and Ellenberg [20] found that chorioamnionitis was not only a predictor of low birthweight but also a contributor to the risk of cerebral palsy in these babies.

This important association raises various hypotheses warranting further investigation. Leviton [21] has suggested that some pregnant women mount an excessive response to infection by means of tumour necrosis factor (TNF), resulting in disturbances to the cerebral vasculature and subsequent ischaemic lesions. McGuire et al [22] established a genetic factor to explain excessive TNF-mediated responses in children with cerebral malaria, and a similar genetically determined factor might have a role in the pathogenesis of cerebral palsy in some preterm babies. TNF stimulates prostaglandin production, [23] and it is possible that a combination of factors, including prostaglandins, contribute to disturbances in the cerebral vasculature of the developing brain. High prostaglandin E_2 concentrations in fetal membranes have been found in association with very early preterm labour when chorioamnionitis is present. [19]

We need to establish whether altering the management of pregnant women with chorioamnionitis affects the likelihood of cerebral palsy, and this can only be done by a randomised controlled trial. The Medical Research Council preterm antibiotic uncertainty study (ORACLE) will test the efficacy of antibiotic therapy for subclinical infection in mothers of preterm babies, but long-term follow-up will be required to detect any changes in cerebral palsy rates.

Pre-eclampsia was associated with a significant decrease in the risk of cerebral palsy in our study. This finding has not previously been documented but the association between pre-eclampsia and a reduced rate of germinal-matrix and intraventricular haemorrhage has been recognised. [10,11,24] Germinal-matrix/intraventricular haemorrhage has in turn been identified as a risk factor for cerebral palsy in two studies of very-low-birthweight babies. [25,26] In addition to a lower frequency of

haemorrhage in such infants born to women with pre-eclampsia, magnesium sulphate given to prevent preterm delivery also seemed to protect against cerebral haemorrhage. [10] Nelson and Grether [12] reported an association between the administration of magnesium sulphate for preterm labour or pre-eclampsia and a reduced risk of cerebral palsy in very-low-birthweight infants. The findings of the Eclampsia Trial Collaborative Group, [27] showing the effectiveness of magnesium sulphate in treating eclampsia, together with those of Nelson and Grether [12] are likely to lead to renewed interest in this treatment for both pre-eclampsia and preterm labour. Randomised controlled trials of magnesium sulphate use in preterm labour and pre-eclampsia should be done before the treatment becomes entrenched in clinical practice. No mothers in our study received magnesium sulphate so we can be confident of the independence of the apparent protective effect of pre-eclampsia. All the babies in the study, however, were delivered without labour, a factor independently associated with a decreased risk of cerebral palsy. A randomised controlled trial, though at present not feasible, would be required to isolate the two effects.

Intrauterine growth retardation resulting in the baby being small for gestational age at birth is a risk factor for cerebral palsy in term babies, [7,28] but not in babies born before 33 weeks of gestation; [27] we confirmed this finding. We can only speculate why poor growth reflecting an adverse intrauterine environment is deleterious later but not earlier in gestation. Perhaps removal of very preterm babies from the intrauterine environment early prevents the effects of chronic deprivation seen in growth-retarded babies at later gestations. Alternatively, the pathological process of intrauterine growth retardation early in gestation might differ from that later in gestation, as may also be the case for pre-eclampsia. It is, however, possible that preterm babies with intrauterine growth retardation die in the neonatal period, thus obscuring the association between this factor and cerebral palsy in survivors.

There has been much interest in establishing the best mode of delivery for very preterm babies. In our study, as in others, [29,30] vaginal delivery with a cephalic presentation was not associated with a significantly increased risk of cerebral palsy. Similarly, vaginal delivery with breech presentation was not associated with a significantly increased risk of cerebral palsy, but this observation must be interpreted cautiously because the number of cases was small. Caesarean section in labour was associated with a significantly increased risk of cerebral palsy, but this association may be due to factors necessitating emergency delivery. Our study could not establish the optimum mode of delivery for women in preterm labour. A well-designed trial is needed. Delivery without labour (elective caesarean section) was independently associated with a decreased risk of cerebral palsy. The explanation for this finding is unclear; there may be unidentified differences between the babies of mothers who undergo labour and those who do not.

Although Apgar scores of 3 or less at 5 min were associated with a

significant increase in the risk of cerebral palsy, the results of cord-blood gas estimates, intrapartum cardiotocography, and late antepartum cardiotocography were inconclusive owing to small numbers. Studies of preterm cerebral palsy will always be limited by the numbers available, however, and we decided to record these data because of the potential for pooling with data from future studies.

Not only do our findings raise several aetiological hypotheses that could be tested, but they also point to possible preventive strategies. For example, it is tempting to speculate that rigorous management of common adverse antenatal factors could lead to a reduction in frequency of cerebral palsy in very preterm babies. This hypothesis could be tested with well-designed randomised controlled trials.

We thank the management group of the Oxford Region Register of Early Childhood Impairment (ORRECI) for permission to use the register; Miss Rosemary King, administrative co-ordinator, for extracting data from the register; and colleagues at the National Perinatal Epidemiology Unit for comments. DJM is funded by Action Research and AMJ by the Department of Health.

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Reactiv Oxygen Speci s Produc d by Macrophag -derived Foam C lls R gulate the Activity of Vascular Matrix Metalloproteinases In Vitro: Implications for Atherosclerotic Plaque Stability

[Articles]

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The results described in this study have been published in abstract form (1996. Circulation. 94:A0099).

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Outline

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Abstract

Vulnerable areas of atherosclerotic plaques often contain lipid-laden macrophages and display matrix metalloproteinase activity. We hypothesized that reactive oxygen species released by macrophage-derived foam cells could trigger activation of latent proforms of metalloproteinases in the vascular interstitium. We showed that in vivo generated macrophage foam cells produce superoxide, nitric oxide, and hydrogen peroxide after isolation from hypercholester-olemic rabbits. Effects of these reactive oxygens and that of peroxynitrite, likely to result from simultaneous production of nitric oxide and superoxide, were tested in vitro using metalloproteinases secreted by cultured human vascular smooth muscle cells. Enzymes in culture media or affinity-purified (pro-MMP-2 and MMP-9) were examined by SDS-PAGE zymography, Western blotting, and enzymatic assays. Under the conditions used, incubation with xanthine/xanthine oxidase increased the amount of active gelatinases, while nitric oxide donors had no noticeable effect. Incubation with peroxynitrite resulted in nitration of MMP-2 and endowed it with collagenolytic activity. Hydrogen peroxide treatment showed a catalase-reversible biphasic effect (gelatinase activation at concentrations of 4 micro M, inhibition at >or=to 10-50 micro M). Thus, reactive oxygen species can modulate matrix degradation in areas of high oxidant stress and could therefore contribute to instability of atherosclerotic plaques. (J. Clin. Invest. 1996. 98:2572-2579.)

Key words: atheroma, extracellular matrix degradation

Abbreviations used in this paper: MMP, matrix metalloproteinase; MMP-2, matrix metalloproteinase-2 or gelatinase A; MMP-9, matrix metalloproteinase-9 or gelatinase B; SMC, smooth muscle cells; X/XO, xanthine/xanthine oxidase.

Introduction

Dysregulated metabolism of extracellular matrix, principally due to focal overexpression of matrix metalloproteinases (MMPs), may contribute to weakening of the atherosclerotic plaque [1-3]. This results in plaque rupture [4], which underlies as many as 90% of acute myocardial infarctions [5]. The regions of the plaques prone to rupture are the "shoulder areas," which often contain macrophages [6,7]. Colocalization of macrophage foam cells and active forms of MMPs within these vulnerable regions is likely relevant for disruption of atherosclerotic lesions [1,8]. While there is a strong connection between plaque vulnerability and the presence of macrophages [6,7,9,10], the mechanisms whereby macrophages influence MMP activity remain poorly defined.

MMP regulation occurs both at the level of gene transcription [11] and activation of proenzymes [12]. Several biological mediators have been noted to induce the expression of MMPs, thereby disturbing the tenuous balance between them and their endogenous inhibitors-the tissue inhibitors of MMPs or TIMPs. MMPs are secreted in a latent, zymogen form in which the prodomain is thought to fold over and shield the catalytic site. This conformation of zymogens is maintained due to thiol interactions between cysteine residues in the prodomain and the zinc atom present in the catalytic site of all MMPs. In vitro, MMP activation can occur when the prodomain is cleaved by other proteases [13-15] or when the zinc-cysteine bond is interrupted [16]. Such an interruption leads to autoactivation [17]. In vivo, the presence of proteolytic activators has not been established definitively in situations where MMPs are clearly active. This raises the possibility that activation by other proteases may not be necessary and that alternate pathways of MMP activation may occur in vivo.

Reactive oxygen species are known to react with thiol groups, such as those involved in preserving MMP latency, so they could modulate the activity of MMPs. The sources of reactive oxygen species in the vasculature are diverse and include vascular smooth muscle cells (SMC) [18], endothelial cells [19,20] and, importantly, macrophages [21]. During the past several years, it has become obvious that an increase in the steady state levels of reactive oxygen species occurs in a number of pathological processes that affect the blood vessels, such as atherosclerosis [22], certain forms of hypertension [23], and diabetes mellitus [24,25]. We hypothesized that in areas of atherosclerotic plaques rich in macrophages activation of extracellular MMPs could occur through interaction with reactive oxygen species. Therefore, we initially defined the nature of reactive oxygen species released by lipid-laden macrophages isolated from tissues of hypercholesterolemic experimental animals. These experiments provided a basis for a rational choice of reactive oxygen species whose effects upon MMP activity were to be tested. Then, we used exogenously generated reactive oxygen species and performed in vitro experiments with latent MMPs produced by vascular SMC.

Methods

Isolation of lipid-laden macrophages from experimental aortic atheroma and carrageenan-induced granuloma

New Zealand White rabbits were fed a 0.5% cholesterol and 4.5% coconut oil diet for 9 wk. To induce granulomas, sterile 1% carrageenan in saline was injected subcutaneously. The injection of carrageenan in hypercholesterolemic rabbits has been shown to induce the formation of macrophages which accumulate intracellular lipid and are indistinguishable from atheromatous foam cells when evaluated by scanning electron microscopy, oil red O, and nonspecific esterase staining [26]. Furthermore, the lipid metabolism and content of macrophage foam cells in granuloma or in aorta of hypercholesterolemic rabbits were found previously to be similar

[27]. Rabbit aortic atheroma was produced by balloon injury as described previously [28]. At the end of the feeding period, the animals were killed using 100 mg/ml pentobarbital.

Lipid-laden, macrophage-derived foam cells were isolated from aortic atheromas and subcutaneous granulomas. Briefly, the aorta and the granulomas were separately harvested, minced, and then incubated under sterile conditions with HBSS containing collagenase (type I; Worthington Biochemical Corp., Freehold, NJ), elastase, and soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) [28]. The resultant turbid fluid was filtered through a sterile nylon mesh filter and cells were collected in sterile tubes. Foam cells were then isolated by metrizamide density centrifugation as described previously [28,29]. Aliquots of cells were resuspended in Opti-MEM (Gibco Laboratories, Grand Island, NY) and used for cell counting and measurement of reactive oxygen species. The presence of intracellular lipid was confirmed by intense Nile red (Molecular Probes, Inc., Eugene, OR) staining of the cytoplasm.

To study non-lipid-laden macrophages, we obtained alveolar macrophages by bronchoalveolar lavage in the same rabbits. After anesthesia, but before death, the trachea was cannulated with sterile plastic tubing (4 mm) and gently lavaged twice with 25 cm^3 of sterile normal saline. The lavages were then centrifuged and pelleted out at 500 g for 5 min. The alveolar macrophages were resuspended in Opti-MEM and then subjected to the same handling procedures as the foam cells (i.e., enzymatic digestion, centrifugation) to check for the possible effect of the isolation protocol on reactive oxygen species production.

The protocol for animal use has been approved by the Emory University Committee on Institutional Animal Care and Use.

SMC culture

SMC were grown from explants of human saphenous veins obtained at bypass surgery. Cells from passages 3 to 4 were grown to confluency in DME (Cellgro/Fisher, Herndon, VA) containing an antibiotic-antimycotic mixture (penicillin 100 U/ml; streptomycin 100 mg/ml; amphotericin B: 0.25 mg/ml; Cellgro/Fisher) and supplemented with 10% FCS. Cells were then washed twice with HBSS and transferred into serum-free medium (DME/F12; 1:1), supplemented with 1 mM insulin and 5 micro g/ml transferrin, for 24 h at the end of which SMC-conditioned culture media containing pro-MMP-2 were harvested. In some experiments, SMC were incubated for 24 h with PMA (100 ng/ml, Sigma Chemical Co.) for induction of pro-MMP-9.

Affinity purification of MMPs

MMP-2 and -9, also called gelatinases due to their activity toward gelatin,

can be isolated based on their affinity for this substrate. Briefly, 1 ml of SMC-conditioned culture medium from unstimulated cells or cells stimulated with PMA (to induce secretion of MMP-9) was added to 100 micro l gelatin-agarose beads (Sigma Chemical Co.) and the mixture was incubated for 1 h at 4 degrees C. The mixture was then centrifuged briefly, washed, and the gelatinases were eluted from the agarose by adding 100 micro l cold 10% DMSO. The mixture was incubated for 5 min and pulse-centrifuged, after which the supernatant containing the eluted gelatinases was loaded onto polysulfone ultrafuge filters (30,000 NMWC; Micron Separations Inc., Westboro, MA) and centrifuged again for 5 min at 3,500 rpm to remove DMSO. The filtered supernatant (containing the MMPs) was subsequently used to study activity of MMP-2 and -9.

Cell-free incubation experiments

In cell-free experiments, we tested the effect of reactive oxygen species on MMPs present either in the conditioned culture medium harvested from SMC or affinity-purified MMPs. Incubations were carried out in a total volume of 500 micro l for times ranging from 30 min to 24 h. To generate O_2 sup.- and H_2O_2, we used mixtures of xanthine and xanthine oxidase (X/XO). In most experiments, culture media or purified gelatinases were incubated with 100 micro M xanthine and 5 mU/ml xanthine oxidase (Sigma Chemical Co.). Under these conditions, 16.52 micro M O_2 sup.- was generated, as measured by lucigenin chemiluminescence. In other experiments, similar samples were incubated with H_2O_2 (Sigma Chemical Co.), at final concentrations varying from 4 to 50 micro M. To examine the effect of nitric oxide, we used the NO donors, S-nitroso-N-acetyl-D, L penicillamine (SNAP) or SPER/NO, (Z)-1-(N-[3-aminopropyl]-N-[4-(3-amino-propylammonio)butyl]-amino)-diazen-1-ium-1,2-(Spermine NONO-ate) (Research Biochemicals Inc., Natick, MA) at 50-500 micro M. Peroxynitrite anion (ONOO sup -, purchased as a 0.17 M stock in 0.3 M NaOH; Alexis Corp., San Diego, CA) was also added directly to culture medium (final concentration of 10-500 micro M). The effect of adding the highest volume of 0.3 M NaOH (vehicle corresponding to the highest concentration of ONOO sup -) to the samples was also examined. Since H_2O_2 and ONOO sup - were not continuously generated and have a short life time, we added repetitive equal doses of these reactive oxygen species in some experiments. At the end of each incubation, samples were directly loaded on to gels for SDS-PAGE zymography or Western blotting.

SDS-PAGE zymography

Proteins with gelatinolytic activity were identified by electrophoresis in the presence of SDS in 10% discontinuous polyacrylamide gels containing 1 mg/ml gelatin. In this method, after electrophoretic migration, proteins with gelatinolytic activity can be detected due to their capacity to digest the

gelatin substrate incorporated into SDS-PAGE gels. Culture media were loaded on gels directly or after affinity purification. The proteins in the gels were renatured by exchanging SDS with Triton X-100 (two 15-min incubations with 2.5% Triton X-100). The gels were subsequently incubated overnight at 37 degrees C in 50 mM Tris-HCl, pH 7.4, containing 10 mM CaCl sub 2 and 0.05% Brij 35. At the end of the incubation, gels were stained with Colloidal Brilliant Blue G (Sigma Chemical Co.). Proteins having gelatinolytic activity were then visualized as areas of lytic activity on an otherwise blue gel. Migration of proteins was compared with that of prestained low molecular weight range markers (Bio-Rad, Hercules, CA). Identical gels were incubated in parallel in the presence of 0.01 M EDTA. Disappearance of lytic bands in these gels confirmed the metal dependence of gelatinolytic activity characteristic of MMPs. Photographs of the gel were scanned by an imaging densitometer and quantified using the NIH Image 1.55 software program.

Enzymatic assays

The type IV collagenase activity of SMC-conditioned culture medium after incubation with different reactive oxygen species was detected using ^{3}H-collagen type IV (Du Pont-NEN, Wilmington, DE). Aliquots (50 and 100 micro l) of either treated or untreated SMC culture medium were incubated with 2 micro g ^{3}H-collagen IV (specific activity 0.14 mCi/mg). Some samples were incubated with treatments alone (reactive oxygen species or reactive oxygen species-generating systems) to test their direct effect upon degradation of collagen IV. All samples were incubated at 37 degrees C for 18 h, then reactions were stopped by addition of reducing SDS-PAGE sample buffer and samples were boiled for 10 min and loaded onto 10% SDS-polyacrylamide gels. Degradation of radiolabeled collagen was assessed by fluorography of gels dried after impregnation with EN^{3}HANCE (DuPont-NEN).

Western blotting

SMC culture media were separated on 10% SDS-PAGE mini gels and transferred onto nitrocellulose membranes (Bio-Rad), using a semidry blotting system (Bio-Rad). Blocking of nonspecific binding was achieved with incubation of the membrane in 5% milk in 50 mM Na phosphate buffer, pH 7.2, 150 mM Na chloride (PBS) containing 0.1% Tween 20. Rabbit polyclonal antibodies to MMP-2 were obtained from Dr. William Stetler-Stevensen (National Institutes of Health, Bethesda, MD). Monoclonal antibodies against nitro-tyro-sine were kindly provided by Dr. Joseph S. Beckman (University of Alabama at Birmingham). Antigen detection was performed with a chemiluminescent detection system as per the manufacturer's instructions (ECL[TM], Amersham International, Buckinghamshire, UK).

Reactive oxygen species produced by macrophages

O_2 sup. production. O_2 sup.- production by macrophages was measured by lucigenin chemiluminescence. Lucigenin is a sensitive and specific measure of O_2 sup.- release [30]. Details of this assay have been published previously [20,22]. Scintillation vials containing 250 mM lucigenin solution in 50 mM phosphate buffer (pH 7.4) were placed in a scintillation counter in the out-of-coincidence mode and allowed to dark adapt for 4 min to obtain background counts. After counting the number of cells, 10^6 macrophages were suspended in 50 mM phosphate buffer and added to the vial containing lucigenin. Counts were obtained at 1-min intervals for 12 min. Steady state counts corrected for the back ground values were then expressed as counts/10^6 cells/min. To study the effect of exogenous stimuli, macrophages were incubated with PMA (final concentration 100 micro M) for 5 min before measurement of O_2 sup.-.

Measurement of NO synthase activity by conversion of L-[sup 14 C]arginine to L-[sup 14 C]citrulline. Freshly isolated macrophages were homogenized in the presence of protease inhibitors (1 mM pepstatin A, 2 mM leupeptin, 1 mM bestatin, and 1 mM PMSF). NO synthase activity was assayed in the particulate fraction of the homogenate. Each sample, normalized by protein (100 micro g), was incubated in the presence of cofactors [final concentration: 100 nM calmodulin, 2.5 mM $CaCl_2$, 1 mM NADPH, 3 mM tetrahydrobiopterin] and the substrate 100 mM/liter L-arginine combined with L-[2,3-sup 3 H]arginine (0.2 mCi; specific activity, 55 Ci/mmol) for 15 min at 37 degrees C. The mixture also contained 1 mM L-citrulline to minimize any conversion of the formed L-[2,3-sup 3 H]citrulline back to L-[2,3-sup 3 H]arginine [31]. After the incubation period, the reaction was quenched by addition of 1 ml of stop buffer (20 mM Hepes, pH 5.5; 2 mM EDTA, and 2 mM EGTA). The reaction mix was applied to a 1-ml column that had been preequilibrated with the stop buffer. Radioactivity associated with L-[2,3-sup 3 H]citrulline was eluted twice from a Dowex AG 50WX-8 column (Na sup + form, Bio-Rad) and measured by liquid scintillation counting.

Detection of H_2 O_2 production by 2,7-dichlorofluorescein (DCF) fluorescence. This test is based on the H_2 O_2-mediated conversion of 2,7-dichlorofluorescein diacetate (DCFA; Molecular Probes, Inc.) into fluorescent DCF, with increased fluorescence emission reflecting enhanced H_2 O_2 production [32]. Briefly foam cells in tissue culture dishes were loaded with 10 mM DCFA by incubation for 30 minutes. Before analysis, cells were washed two times with PBS and then imaged by confocal scanning laser microscopy using 488 nm excitation and 510 nm emission filters.

Results

Reactive oxygen species production in lipid-laden macrophages Superoxide production. We found that lipid-laden macrophages isolated from either atherosclerotic aortas or granulomas of hypercholesterolemic rabbits generated O_2 sup.- (Figure 1 A). Counts reflecting O_2 sup.- production were higher in lipid-laden macrophages than those generated by non-lipid-laden alveolar macrophages from the same animals (e.g., Figure 1 A, steady state chemiluminescence levels of 153.7 +/- 13.0 and 81.1 +/- 5.2 from foam cells vs. 31.7 +/- 5.6 x 10^3 counts/min/10^6 alveolar macrophages, respectively). Interestingly, aortic foam cells, in the absence of exogenous stimulation, produced even more O_2 sup.- than alveolar macrophages stimulated with PMA (153.7 +/- 13.0 vs. 86.0 +/- 4.3 x 10^3 counts/min/10^6 cells, respectively). The level of O_2 sup.- production was increased severalfold by PMA stimulation in both aortic (from 153.7 +/- 13.0 x 10^3 counts/min/10^6 cells to 561.5 +/- 9.0 x 10^3 counts/min/10^6 cells) and in granuloma-derived foam cells (from 81.1 +/- 5.2 to 616.4 +/- 25.0 x 10^3 counts/min/10^6 cells), suggesting a high oxidative potential.

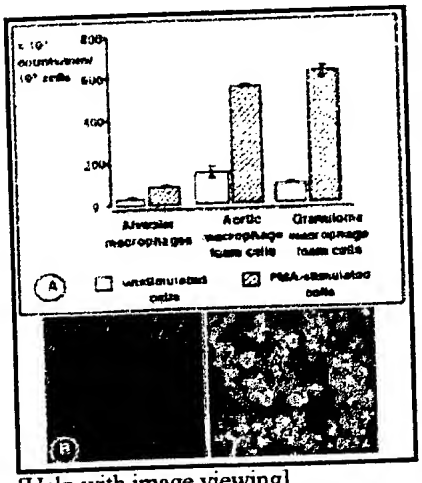

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Figure 1. (A) Lucigenin-enhanced chemiluminescence, reflecting production of superoxide by alveolar macrophages, aortic foam cells, and granuloma foam cells isolated from cholesterol-fed rabbits. Unstimulated cells as well as cells stimulated with PMA were assayed. The figure shows the average of triplicate measurements of steady state levels of chemiluminescence 15 min after adding cells to the lucigenin-containing buffer. In this assay, 10 pmol of O_2 sup.- generated from known amounts of xanthine and xanthine oxidase yielded [approximately] 4,700 counts. (B) Detection of hydroperoxides as shown by the fluorescence of DCF in lipid-laden macrophages. The left panel shows the autofluorescence of cells incubated in the absence of DCFA and the right panel shows cells 30 min after addition of DCFA. Cells were imaged with a confocal microscope using a magnification of 40 and an FITC filter.

NOS activity. NOS activity in foam cells from several granulomas was highly variable. In some, as little as 0.26 +/- 0.03 pmol/mg protein/min of ^{3}H-citrulline was detected, while in others as much as 4.7 pmol/mg protein/min of sup 3 H-citrulline was formed. In each preparation NOS - activity was not affected by addition of EGTA (average^3 H-citrulline in the presence of EGTA was 0.82 +/- 0.30 pmol/mg protein/min), suggesting that the NO synthase responsible for this activity was the inducible isoform. Due to limited cell yield for aortic foam cells, we measured formation of^3 H-citrulline only in a pair of samples obtained from one experiment, in

which cells from four rabbits were pooled. This was 1.32 pmol/mg protein/min. Alveolar macrophages isolated from the same rabbits had no detectable NO synthase activity.

$H_2 O_2$ detection. Lipid-laden macrophages cultured for 24 h, then incubated with DCFA (10 micro M), showed intense fluorescent staining by confocal microscopy, indicative of peroxide generation (Figure 1 B).

Effect of reactive oxygen species on vascular MMP activity in vitro

X/XO system activates latent MMPs. Incubation of human vascular SMC culture media containing latent MMP-2 (pro-MMP-2) with X/XO (100 micro M:5 U/liter) resulted in activation of pro-MMP-2. Activation also occurred when culture media were incubated with X/XO after harvesting from tissue culture dishes. To rule out the contribution of other components in the culture media that could serve as intermediaries in this activation sequence, latent MMP-2 and MMP-9 (induced by PMA treatment of vascular SMC) were purified using gelatin-agarose. The purified gelatinases were then exposed to the X/XO system, which led again to activation of pro-MMP-2 as well as pro-MMP-9 (Figure 2). Figure 2 presents the typical appearance of gelatinolytic bands with lower molecular weights after incubation with X/XO. XO by itself had no effect upon latent MMP-2 (data not shown), but was responsible for production of lytic bands that migrate close to the bottom of 10% gels (Figure 2) and are not inhibited by incubation with EDTA. Xanthine neither affected pro-MMP-2 nor produced any lytic pattern in gelatin gels, suggesting that pro-MMP-2 activation was due to a product of the X/XO system. In additional experiments, we tested the effect of adding SOD to the incubates (not shown), but activation of pro-MMP-2 by X/XO was not inhibited, probably due to the simultaneous generation of other reactive oxygen species ($H_2 O_2$ and/or hydroxyl, [centered dot] OH).

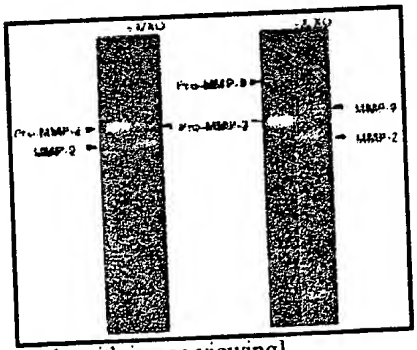

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Figure 2. SDS-PAGE zymography showing the effect of reactive oxygen species generated by X/XO (100 micro M:5 mU/ml) on activity of latent gelatinases, which were affinity-purified from culture medium harvested from human vascular SMC. White areas of lysis produced in the gel, which contained 1 mg/ml gelatin, shows presence of activated, as well as latent, forms of gelatinases. The left panel illustrates activation of latent MMP-2 (pro-MMP-2), purified from the conditioned medium of unstimulated human SMC, after incubation with X/XO. The right panel shows activation of pro-MMP-2 and latent MMP-9 (pro-MMP-9) isolated from medium of SMC stimulated with PMA (which induces MMP-9 expression). Treatment with X/XO resulted in the conversion of latent forms of both gelatinases to the active, lower molecular weight forms (MMP-2 and MMP-9).

Effect of $H_2 O_2$ on MMP-2 activity. $H_2 O_2$ is likely produced by lipid-laden macrophages in vivo. In vitro, $H_2 O_2$ is a main product of the X/XO system, which we used to generate reactive oxygen species in vitro, and thus could have contributed to the activation of MMP-2 that we observed using X/XO. The direct effect of $H_2 O_2$ on pro-MMP-2 activity was tested by incubating aliquots of cell culture media with various concentrations of $H_2 O_2$. At low concentrations, incubation with $H_2 O_2$ increased gelatinolysis associated with the activated form of MMP-2 and also induced generation of lower molecular weight gelatinolytic bands (Figure 3 A). Higher doses of $H_2 O_2$ (10-50 micro M) resulted in concentration-dependent inactivation of gelatinolytic activity (Figure 3 A, and Figure 3 B). The effects of $H_2 O_2$ could be blocked completely by addition of catalase (Figure 3 A). We confirmed that $H_2 O_2$ initiates molecular processing of latent MMP-2 by identifying the lower molecular species with anti-MMP-2 antibodies (shown in Figure 6). Compared with control culture media, aliquots incubated with $H_2 O_2$ displayed increased immunoreactivity of lower molecular weight species and additional immunoreactive bands.

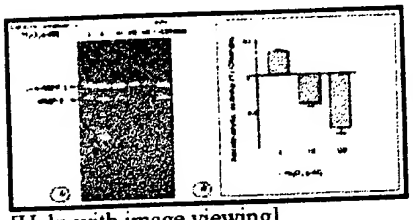

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Figure 3. (A) SDS-PAGE zymography of media conditioned by human SMC. Identical aliquots were exposed to various concentrations of $H_2 O_2$ for 4 h. The upper arrow points to the latent form of MMP-2 (pro-MMP-2). Notice that incubation with 4 micro M $H_2 O_2$ activated pro-MMP-2 to the lower molecular weight active form MMP-2 (lower arrow) and even to smaller forms with gelatinolytic activity. Higher doses of $H_2 O_2$ led to inactivation (disappearance of gelatinolytic activity). The effect of the highest concentration of $H_2 O_2$ (50 micro M) was prevented by addition of catalase (250 U/ml). (B) Effect of $H_2 O_2$ upon the gelatinolytic activity associated with the active form of MMP-2, as measured from SDS-PAGE zymography gels. Areas of lysis have been measured by laser scanning densitometry and plotted as the percentage of gelatin lysis produced by control. Data shown have been collected from four independent experiments. Compared with control samples (no $H_2 O_2$), gelatinolytic activity was increased by incubation with 4 micro M $H_2 O_2$, and decreased by incubation with 50 micro M.

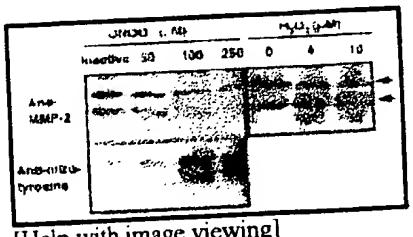

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Figure 6. Immunoblotting of SMC culture media incubated with peroxynitrite (ONOO sup -) or H_2O_2. The top panels show detection of latent MMP-2 (top arrow) and activated MMP-2 (bottom arrow). The bottom left panel shows detection of nitrotyrosine residues using specific antibodies. The membrane containing ONOO sup --treated culture media, presented in the left two panels, was first blotted with anti-MMP-2 antibodies (top left panel), then stripped, and reprobed with antinitrotyrosine antibodies (bottom left panel). Incubation with ONOO sup - led to a decrease in immunoreactivity to MMP-2 antibodies and to an increased immunoreactivity to antinitrotyrosine antibodies. In contrast incubation of culture media with H_2O_2 actually increased the signal associated with activated MMP-2 (bottom arrow), as detected by anti-MMP-2 antibodies.

Effect of sup . NO and peroxynitrite (ONOO sup -) on MMP-2 activity. Generation of sup . NO by SNAP and Spermine NONO-ate (50-500 micro M) in the culture media had no discernible effect upon activation of MMP-2 zymogen (data not shown). However, since the simultaneous production of sup . NO and O_2 sup . - in vivo may lead to the generation of ONOO sup - [33], we also tested the effect of this reactive oxygen species upon MMP-2 produced by cultured vascular SMC. We incubated cells in culture with ONOO sup - and analyzed culture media by SDS-PAGE zymography. We found that after incubation with ONOO sup - the intensity of the MMP-2-associated lytic band, migrating at [approximately] 60 kD, was increased and was accompanied by the appearance of several lower molecular weight gelatinolytic bands (Figure 4). Initially, we suspected that in addition to activation of pro-MMP-2, incubation of SMC with ONOO sup - may induce the production and release of new proteins with gelatinolytic activity. However, the same effect was achieved by incubating the culture media after harvesting from unstimulated SMC (cell-free experiments) with ONOO sup - (Figure 4). This showed that the generation of several gelatinolytic bands running at apparent molecular masses < 60 kD did not require the presence of cells and was likely due to sequential extracellular processing of MMP-2. Additional confirmation that incubation with ONOO sup - results in activation of latent MMP-2 was obtained through a collagenolytic assay using radiolabeled type IV collagen, a typical MMP-2 substrate. Latent MMP-2 incubated with ONOO sup - displayed enzymatic activity against collagen IV (Figure 5). Culture media incubated with pH-inactivated ONOO sup -, containing the stable decomposition products NO_2 sup - and NO_3 sup -, had no collagenolytic activity. The activation of pro-MMP-2 by ONOO sup - was accompanied by tyrosine nitration of MMP-2 (Figure 6), as shown by consecutive immunodetection of MMP-2 followed by detection of nitro-tyrosine residues in samples of SMC culture media. Incubation of culture media with 100-250 micro M of ONOO sup - abolished the immunoreactivity of the anti-MMP-2 antibody for bands associated with activated forms of MMP-2, while increasing the

immunoreactivity of antinitrotyrosine antibodies for the corresponding bands. This effect suggested that, during activation, the MMP-2 epitopes recognized by the MMP-2 antibody were modified by nitration. Further supporting the hypothesis of a specific modification of MMP-2 by ONOO sup - treatment was the finding that the same anti-MMP-2 antibody recognized an MMP-2 form activated by H sub 2 O_2 (Figure 6).

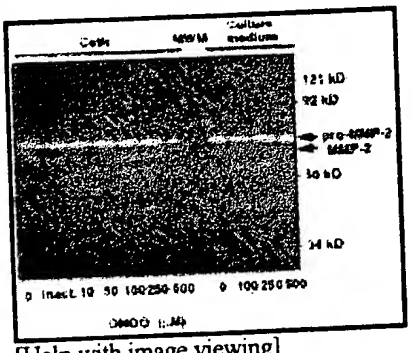

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Figure 4. Effect of peroxynitrite on gelatinolytic activity in culture media of human SMC. In left lanes, we applied culture media from SMC incubated with increasing concentrations of ONOO sup - for 24 h. One cell culture dish was incubated with the highest concentration of pH-inactivated ONOO sup - (Inact.). The right lanes show activity in media harvested from unstimulated SMC, then incubated with similar concentrations of ONOO sup -. The same gelatinolytic pattern was obtained in both cases (+/- SMC). Molecular weight of prestained markers (MWM), loaded in between samples obtained from the two experiments, is indicated at right in kiloDaltons (kD).

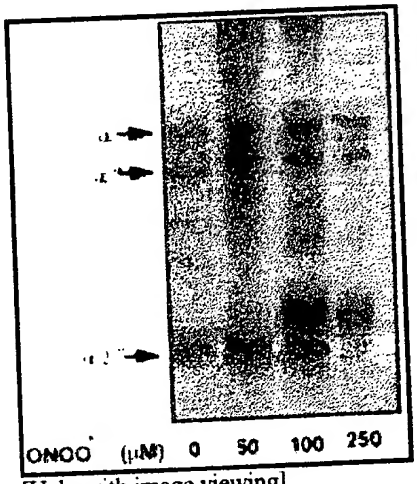

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Figure 5. Fluorographic image of ^{3}H-type IV collagen incubated with SMC-conditioned medium treated with ONOO sup -. Typical collagen bands are indicated with arrows. Treatment with ONOO sup - imparts collagenolytic activity to SMC-derived culture media, as suggested by the diminishing radio-activity associated with alpha 2 double prime chains and proteolytic processing of other higher molecular weight collagen chains.

Discussion

This study was undertaken to examine the possibility that reactive oxygen species produced by lipid-laden macrophages modulate vascular MMP activity. We found that macrophage foam cells, isolated from atheroma, steadily produce O_2 sup . -, H_2 O_2, and sup . NO without additional exogenous stimulation. We also showed that these reactive oxygens can modulate MMP-2 and -9 activity, either directly or via a derivative radical, ONOO sup -. This may be an important mechanism for modulating MMP activity in atherosclerotic plaques. Similar radicals may also be responsible

for MMP activation in inflammation or other conditions associated with oxidative stress.

Traditionally, the theory regarding MMP activation in vivo postulates the attack of susceptible regions in the propeptide MMP domain by soluble proteases [13], or by a membrane-bond protease in the case of MMP-2 [34,35]. Studies from Van Wart's group [16] led to the formulation of the "cysteine-switch" hypothesis to explain the seemingly disparate means by which activation could be achieved in vitro by a wide variety of agents that share one common characteristic: thiol reactivity. In this model, the zinc atom at the active site is coordinately bound to an unpaired cysteine thiol group located approximately at the 80^{th} residue of the propeptide domain. Disruption of this interaction is believed to represent the critical step in initiating the process of MMP autoactivation. Reactive oxygen species undergo facile reactions with thiol groups and may serve as a common mechanism of activation for several different MMPs. Macrophage-derived reactive oxygen species may thus provide a link between the presence of activated forms of MMPs and macrophage-rich areas in atherosclerotic plaques.

Rosenfeld et al. showed previously that macrophage-derived foam cells isolated from hypercholesterolemic rabbit aortas are capable of oxidatively modifying lipoproteins [29], but the nature of reactive oxygen species released by these cells has not been defined. To our knowledge, all other previous studies have examined reactive oxygen species production by monocytes differentiated in vitro and subsequently activated with exogenous stimuli. Properties of cells differentiated and loaded with lipid in vitro may differ significantly from foam cells generated in vivo. In this study, we examined two types of macrophages that accumulate lipid in vivo, in tissues of hypercholesterolemic animals. By producing lipid-laden macrophages in granulomas we have generated large quantities of foam cells for study, in contrast to aortic foam cells isolated from atheroma, where the yield of isolated cells is low. Further, rapid isolation (< 4 h from time of killing of the animal) precludes the influence of extraneous factors that may influence radical production.

Macrophage foam cells, whether derived from aortic atheroma or granuloma of hypercholesterolemic animals, produced substantial amounts of superoxide compared with non-lipid-laden macrophages obtained from the same animal. Lipid-laden macrophages expressed varying amounts of NO synthase activity. In contrast, NO synthase activity was undetectable in alveolar macrophages. These findings provided a basis for experiments testing the effects of O_2 sup . -, H_2 O_2, sup . NO, and ONOO sup - on activity of vascular MMPs. To directly examine the effects of these, we either generated or exogenously added the various reactive oxygen species of interest. MMPs investigated were produced by cultured human SMC. These cells can produce a spectrum of MMPs [36] and likely contribute substantially to the MMPs produced in human vessels.

We concentrated on vascular gelatinases for two reasons. First, MMP-2 is ubiquitous in normal and atherosclerotic human vessels [1] and is produced in vitro by vascular human endothelium [37] and SMC [36]. Generation of activated forms of MMP-2 is associated with SMC migration and proliferation in vitro and in vivo in animal models of restenosis [38-41]. Induction of MMP-9 also occurs under these conditions [38-41]. Pro-MMP-9, the main MMP product of monocyte-macrophages [42], is expressed in human atheroma [1] and can be detected in specimens collected from patients with unstable angina [43]. Second, the biochemical characteristics of these two gelatinases, which have prodomains rich in cysteine residues, would presumably increase their susceptibility to the action of reactive oxygen species.

The X/XO reaction, commonly used to generate O_2 sup . -, also generates other reactive oxygen species: X/XO can reduce dioxygen univalently to O_2 sup . - or divalently to $H_2 O_2$. The balance between these two pathways depends on the state of reduction of the enzyme, favoring divalent reduction when the enzyme is fully reduced. Conditions such as substrate concentration, pO_2, and pH affect the reduction state of the enzyme. Thus, it is possible that, in our experiments with the X/XO system, gelatinases were subjected to the action of reactive oxygen species other than O_2 sup . -. Furthermore, addition of SOD did not block activation of MMP-2 in the presence of X/XO, which could mean that a radical other than O_2 sup . - may be involved. $H_2 O_2$ generated by X/XO or through dismutation of O_2 sup . - could have reacted with pro-MMP-2. We tested this hypothesis and found that direct addition of $H_2 O_2$ caused modulation of MMP-2 activity. Activation of MMP-2 at low doses of $H_2 O_2$ (4 micro M) and inactivation at higher doses (10-50 micro M) suggest that there may be a biphasic response to oxidant stress. High concentrations of $H_2 O_2$ may inhibit MMP-2 enzymatic activity through mobilization of the zinc atom at the catalytic site [44]. Also, higher $H_2 O_2$ concentrations may modify the zymogen more extensively, leading to protein degradation, and could act as a regulatory mechanism for MMP activity. Similarly, the highest concentration of ONOO sup - that we used (500 micro M) seemed to inactivate pro-MMP-2. We cannot exclude the possibility that, in our in vitro conditions, the OH sup - radical was also formed via the $H_2 O_2$-dependent Fenton reaction which could be supported by free metals in the culture medium.

This is, to our knowledge, the first demonstration of a protein being activated by ONOO sup -. ONOO sup - has strong oxidizing properties that may be responsible for free radical-dependent toxicity in atherosclerosis and other pathological conditions [45-47]. ONOO sup - has the ability to nitrate tyrosine residues of proteins [48-51]. Our results showed that ONOO sup -

activates pro-MMP-2. Incubation with inactivated ONOO sup -, containing its decomposition products NO_3 sup - and NO_2 sup -, had no effect on the activation of MMP-2. It has been estimated that the average rate of ONOO sup - formation may reach 1 mM/min in the rat lung epithelial fluid upon stimulation of alveolar macrophages [52]. The concentrations used in our study are thus within the range found in pathophysiologic states. Immunoblotting with specific antibodies showed that activation of MMP-2 was associated with almost exclusive tyrosine nitration of proteins migrating at [approximately] 70 kD (likely MMP-2) and concomitant loss of reactivity to anti-MMP-2 antibodies. It is interesting to speculate that nitration of two tyrosine residues, present within a five amino acid stretch in the hinge region between the propeptide and active domains of pro-MMP-2, could assist in unfolding of the zymogen. Using the same antinitrotyrosine antibody to stain atherosclerotic lesions, Beckman et al. [48] demonstrated an intense immunopositive reaction. Therefore it is possible that nitration of MMPs could contribute to this immunostaining pattern in atheroma. Importantly, the reaction with ONOO sup - generated around activated macrophages provides a possible mechanism by which MMP activity previously detected in vulnerable areas of the atherosclerotic plaque [1,8] is unleashed. sup . NO donors used in our experiments had no effect on MMP-2 activation. A recently published study suggested that sup . NO activates pro-MMP-2 [53]. However, it is possible that ONOO sup -, rather than sup . NO, was directly responsible for activation of MMP-2 in that study, in which cells were simultaneously incubated in culture with cytokines, lipopolysaccharide, and sup . NO donors. Under these conditions both sup . NO and O_2 sup . - may have been generated, leading to formation of ONOO sup - [33].

Macrophage-derived foam cells express MMP mRNA [54], produce MMP proteins [1,28], and colocalize with MMP activity in human and experimental atherosclerotic plaques [1,8]. Shah et al. [3] showed that MMPs secreted by monocytederived macrophages may be responsible for breakdown of tissue collagen by incubating these cells, or their conditioned culture medium, with explanted atherosclerotic lesions in vitro. Since the presence of cells enhanced collagenolysis, it is possible that generation of active MMPs depended on the action of cell-released reactive oxygen species. These reactive species have a short life time and their effects would otherwise rapidly diminish in conditioned culture media.

Based on the findings of this study and of previously published reports, it appears that macrophages may be able to participate in MMP matrix degradation at several levels. These include: (a) inducing MMP expression in other cells (via secretion of cytokines); (b) producing MMPs; and (c) activating latent forms of secreted MMPs (via production of reactive oxygen species). It is of note that antiprotease inhibitors (serpins) are susceptible to degradation by MMPs [55,56], possibly leading to an increase in proteolytic activity in the vicinity of activated macrophages. Similarly, activated neutrophils, which generate hypochlorous acid through the action

of myeloperoxidase, seem capable of autoactivating their latent collagenase [57]. Since myeloperoxidase is also present in atherosclerotic vessels [58], hypochlorous acid may contribute to activation of MMPs and inactivation of serpins.

The results of this investigation provide support for a mechanism by which macrophage-derived foam cells could activate MMPs in the atherosclerotic plaque. Activation of latent MMPs by reactive oxygen species may also be relevant in other pathological conditions associated with high oxidative stress. Our observations may explain some of the benefits of antioxidant therapy [59,60] and may help direct future interventions to improve plaque stability.

Acknowledgments

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A role for matrix metalloproteinase-9 in spontaneous rupture of the fetal membranes

[Transactions Of The Eighteenth Annual Meeting Of The Society Of Perinatal Obstetricians-Continued]

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Outline

Graphics

Abstract

OBJECTIVES: Preterm premature rupture of fetal membranes is responsible for 30% to 40% of preterm deliveries. Fetal membranes are composed primarily of collagen. Matrix metalloproteinases are enzymes capable of degrading

extracellular matrix macromolecules, including collagens. Expression of matrix metalloproteinase-9 (gelatinase B, 92 kd) and its tissue inhibitor (tissue inhibitor of metalloproteinase-1) has been localized in amnion and chorion. The objective of this study was to determine whether rupture of fetal membranes and intrauterine infection are associated with changes in the expression of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1.

STUDY DESIGN: Two hundred one women in the following categories had amniotic fluid retrieved: (1) preterm labor and intact membranes in the presence (n = 42) or absence (n = 21) of microbial invasion of the amniotic cavity, (2) preterm premature rupture of the membranes with (n = 29) or without (n = 23) microbial invasion of the amniotic cavity, and (3) term gestation with intact membranes (n = 50) or with premature rupture of the membranes (n = 40). Women in groups 1 and 2 were matched for gestational age at amniocentesis. Microbial invasion of the amniotic cavity was defined by a positive amniotic fluid culture for micro-organisms. Matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 were measured with use of sensitive and specific immunoassays that were validated for amniotic fluid.

RESULTS: Spontaneous rupture of membranes at term is associated with a significant increase in the amniotic fluid concentrations of matrix metalloproteinase-9 (premature rupture of membranes, no labor: median 3.9 ng/mL, range 2.7 to 11.1 ng/mL vs no premature rupture of membranes, no labor: median <0.4 ng/mL, range <0.4 to 22.4 ng/mL; P < .001). Patients with preterm premature rupture of the membranes had higher median matrix metalloproteinase-9 concentrations than those with preterm labor and intact membranes who were delivered at term (7.6 ng/mL, range <0.4 to 230.81 ng/mL vs 54.5 ng/mL, range <0.4 to 1650 ng/mL; P = .06). Women with microbial invasion of the amniotic cavity had higher median matrix metalloproteinase 9 concentrations than did those without microbial invasion regardless of membrane status (preterm labor: 54.5 ng/mL, range <0.4 to 3910 ng/mL vs <0.4 ng/mL, range <0.4 to 1650 ng/mL; P < .01; preterm premature rupture of membranes: 179.8 ng/mL, range <0.4 to 611 ng/mL vs 7.6 ng/mL, range <0.4 to 230.81; P < .001).

CONCLUSION: Our data support a role for matrix metalloproteinase-9 in the mechanisms responsible for membrane rupture in term and preterm gestations. (Am J Obstet Gynecol 1998;179:1248-53.)

Key words: Premature rupture of the membranes, matrix metalloproteinases, gelatinase B, tissue inhibitor of metalloproteinase, amniotic cavity, amniotic fluid, chorioamnionitis, prematurity

The fetal membranes normally rupture after the onset of spontaneous labor. Premature rupture of the membranes occurs in 10% of women at term and is associated with 30% to 40% of preterm deliveries. [1] The pathophysiologic mechanisms underlying premature rupture of the membranes are poorly understood. [1-4] Histologic studies of the site of rupture have demonstrated a zone of altered morphologic features characterized by thickening of the connective tissue components of the membranes, thinning of the cytotrophoblast layer and decidua, and disruption of the interconnection between amnion and chorion. [5] A significant decrease in the density of collagen types I, III, and V has been reported in the zone of altered morphologic structure. This zone has been localized in close proximity to the rent before the onset of labor, implying that these developments precede the onset of labor. Increased

intrauterine pressure during labor is likely to exert pressure over this weakened area of the membranes, resulting in membrane rupture. Preliminary observations by Bell and Malak [5] suggest that the changes in the zone of altered morphologic structure are more extensive in the setting of preterm premature rupture of the membranes. Thus the current hypothesis is that increased protease activity leads to an accelerated degradation of the extracellular matrix and subsequent membrane rupture. [4,6] In the context of infection, proteases produced by bacteria could decrease membrane strength and elasticity. [7] Alternatively, the proteases could be derived from host cells (eg, macrophages, granulocytes, etc) in response to activation by microbial products.

Collagen types I, III, and V form the major extracellular component of the fetal membranes. Type IV collagen is the major component of the basement membrane and is also present in the spongy and reticular layers of human fetal membranes. [8]

Matrix metalloproteinases are a family of potent enzymes that degrade the extracellular matrix macromolecules, including the collagens. [9,10] Matrix metalloproteinase-1 (MMP-1) (interstitial collagenase) degrades collagen types I, II, and III, whereas degradation of type IV collagen is carried out by the type IV collagenases/gelatinases matrix metalloproteinase-2 (MMP-2) (72 kd gelatinase A) and matrix metalloproteinase-9 (MMP-9) (92 kd gelatinase B). [9,10] MMP-9 additionally degrades collagen type V and elastin. MMP-2 also acts on collagen types V, VII, and X and on elastin. [9-13] The activity of matrix metalloproteinases is regulated by specific tissue inhibitors. The expression of a number of matrix metalloproteinases (including MMP-1, MMP-2, matrix metalloproteinase-3, and MMP-9), as well as tissue inhibitor of metalloproteinase-1 (TIMP-1) and tissue inhibitor of metalloproteinase-2 (TIMP-2), has been demonstrated in human fetal membranes. [14-18] Expression of MMP-9 is increased in fetal membranes during labor and infection. [19,20] Increased activity of MMP-9 in amniotic fluid has been documented in women with preterm premature rupture of membranes. [21]

MMP-9 is capable of degrading type IV collagen, the major collagen component of the amniotic basement membrane. MMP-9 has recently been implicated in premature rupture of membranes and parturition by several investigators. [16,21] The objectives of this study were to determine (1) whether amniotic fluid concentrations of immunoreactive MMP-9 are increased in women with premature rupture of membranes both at term and in preterm gestations, (2) whether microbial invasion of the amniotic cavity results in increased bioavailability of these enzymes in amniotic fluid, (3) whether concentrations of TIMP-1 are altered in these clinical conditions (rupture of membranes and microbial invasion of the amniotic cavity), and (4) the relationship between the immunoreactive concentrations of MMP-9 and enzymatic activity in amniotic fluid, as measured by zymography.

Material and meth ds

Study design. A cross-sectional study was designed to determine the effect of microbial invasion of the amniotic cavity and premature rupture of the membranes on the amniotic fluid concentrations of MMP-9 and TIMP-1. This study included women in the following categories.

Group 1 (n = 52) consisted of women with preterm premature rupture of the membranes, which was defined as amniorrhexis before the onset of spontaneous labor. Membrane rupture was diagnosed with the use of vaginal pooling, ferning, or Nitrazine tests. Preterm premature rupture of membranes was defined as membrane rupture before the onset of spontaneous labor before 37 weeks' gestation. This group was subdivided into women with (n = 29) and without microbial invasion of the amniotic cavity (n = 23). Microbial invasion was defined as a positive amniotic fluid culture for microorganisms.

Group 2 (n = 63) consisted of women in preterm labor with intact membranes. These women were subdivided on the basis of the response to tocolysis and the presence of microbial invasion of the amniotic cavity into (1) women who received tocolysis and were delivered at term (n = 21), (2) women who received tocolysis and were delivered preterm in the absence of documented microbial invasion (n = 21), and (3) women in preterm labor with microbial invasion who delivered a premature infant (n = 21). Preterm labor was defined as the presence of regular uterine contractions occurring with a minimum frequency of 2 every 10 minutes accompanied by changes in cervical effacement or dilation before 37 weeks of gestation.

Group 3 (n = 86) consisted of women at term (ie, >37 weeks' gestation). These women were subdivided into the following groups: (1) women with intact membranes not in labor at the time of amniocentesis (n = 25), (2) women in labor with intact membranes (n = 25), and (3) women with premature rupture of membranes at term in the presence (n = 16) and absence (n = 20) of labor.

Patients in Groups 1 and 2 were matched for gestational age at amniocentesis. Women with other medical and obstetric complications (eg, pre-eclampsia, diabetes) were excluded, as were any women who had anomalous fetuses. The indications for amniocentesis in patients with preterm labor and premature rupture of the membranes were the evaluation of the microbial state of the amniotic cavity and fetal lung maturity. Women at term not in labor underwent amniocentesis for assessment of lung maturity before cesarean section, whereas those in labor underwent amniocentesis because of labor at an uncertain gestational age or for the diagnosis of microbial invasion of the amniotic cavity. Samples of amniotic fluid were selected by searching our Bank of Biological Materials for patients who met the entry criteria. Many of these samples have been used previously in studies of amniotic fluid cytokines and arachidonic acid metabolites in amniotic fluid. All women provided informed consent before

the collection of amniotic fluid and the study was conducted under institutional review board-approved protocols. The National Institute of Child Health and Human Development institutional review board has approved the use of these samples for research.

Amniotic fluid. Amniotic fluid was collected by transabdominal amniocentesis in all cases. A portion of amniotic fluid was delivered to the laboratory for Gram stain and microbial culture. Cultures for aerobic and anaerobic bacteria, as well as for Mycoplasma species, were obtained. The remainder of the amniotic fluid was centrifuged at 4[degree sign]C for 10 minutes to remove cellular and particulate debris. Aliquots of the supernatant were stored at -70[degree sign]C until assay.

MMP-9 and TIMP-1 quantification. Samples of amniotic fluid were assayed for MMP-9 and TIMP-1 in duplicate with the use of commercially available immunoassay kits (Amersham Life Science, Buckinghamshire, United Kingdom). These assays use a sandwich format for the enzyme-linked immunosorbent assays. Before use on study samples, the assay systems were validated for amniotic fluid with spike and recovery experiments. In these experiments graded amounts of recombinant human MMP-9 and TIMP-1 were added to pooled amniotic fluid. Curves parallel to the standard curve in assay buffer were obtained. Samples were diluted before assay and those in which the optical densities were outside the optical densities of standard curve were reassayed at higher dilutions. The sensitivities of the assays were calculated to be 0.4 ng/mL and 1.08 ng/mL for MMP-9 and TIMP-1, respectively, and the interassay and intra-assay coefficients of variation were 10.9% and 6.4% for MMP-9 and 6.0% and 3.9% for TIMP-1, respectively.

Gel substrate zymography. Gelatin zymography was carried out to determine the activity of MMP-9 in amniotic fluid. Sodium dodecyl sulfate-polyacrylamide gels (7.5% to 10%) that had been precast in the presence of calf serum gelatin (0.1%) were used (Novex, San Diego). Amniotic fluid samples were mixed with sample buffer (Tris-hydrochloric acid, sodium dodecyl sulfate, glycerol, and bromophenol blue). Each gel was run with a molecular weight standard as well as recombinant human MMP-9 (50 ng/mL) standard. Electrophoresis at 4[degree sign]C was followed by 2 washes (30 minutes) in renaturing buffer (Triton X-100 25%) to regenerate gelatinolytic activity. The gels were then incubated at 37[degree sign]C for 18 hours in developing buffer (Tris-base, Tris-hydrochloric acid, sodium chloride, calcium chloride, and Brij 35). Coomassie blue (0.1%) was used to stain the gels, which were then destained to visualize clear lysis bands (representing gelatin digestion). The gelatinolytic bands were quantified by standard densitometry.

Statistical analysis. The concentrations of MMP-9 and TIMP-1 in amniotic fluid were not normally distributed, and therefore nonparametric tests were used: Mann-Whitney and Wilcoxon test for censored observations. The

statistical software packages used were SPSS 7.5 (SPSS, Chicago) and True Epistat (Richardson, Texas).

Results

Spontaneous rupture of membranes at term was associated with a significant increase in the amniotic fluid concentrations of MMP-9 and TIMP-1 (Figure 1). The frequency with which MMP-9 was detectable in women with ruptured membranes was higher than in patients at term with intact membranes (100% [36/36] vs 64% [32/50], P < .001, Figure 1).

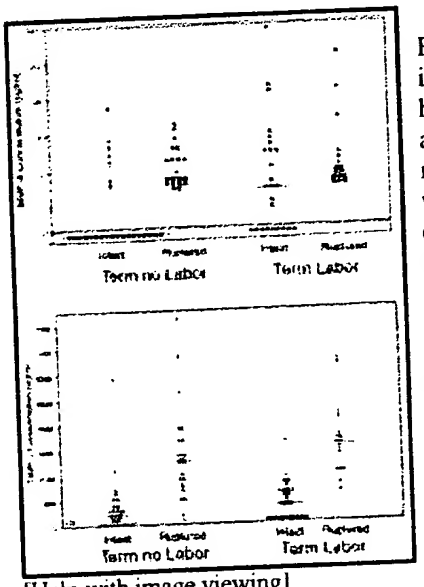

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Figure 1. MMP-9 and TIMP-1 concentrations in amniotic fluid in women at term. Interrupted horizontal line, Sensitivity of assay (0.4 ng/mL and 1.08 ng/mL, respectively). Spontaneous rupture of membranes at term is associated with significant increase in amniotic fluid concentrations of MMP-9 and TIMP-1. MMP-9: Premature rupture of membranes, no labor, median 3.9 ng/mL, range 2.7 to 11.1, versus intact membranes, no labor, median <0.4 ng/mL, range <0.4 to 22.4, P < .001; premature rupture of membranes, labor, median 4.1 ng/mL, range 2.8 to 121.0, versus intact membranes, labor, median 1.6 ng/mL, range <0.4 to 187.3, P < .05. TIMP-1: Premature rupture of membranes, no labor, median 2617.8 ng/mL, range 277.6 to 8025.5, versus intact membranes, no labor, median 250.4 ng/mL, range <1.1 to 5743.6, P < .001; premature rupture of membranes, labor, median 3034.8 ng/mL, range 1172.4 to 6493.2, versus intact membranes, labor, median 739.6 ng/mL, range <1.1 to 3150.1, P < .001. Wilcoxon test for censored observations.

Patients with preterm premature rupture of membranes had higher median MMP-9 concentrations than those with preterm labor and intact membranes who were delivered at term. Moreover, MMP-9 was detectable more often in patients with preterm premature rupture of membranes than in women with preterm labor and intact membranes (88.5% [46/52] vs 61.9% [26/42], P < .01). In contrast, no difference was found between these 2 groups in amniotic fluid concentrations of TIMP-1 (Figure 2).

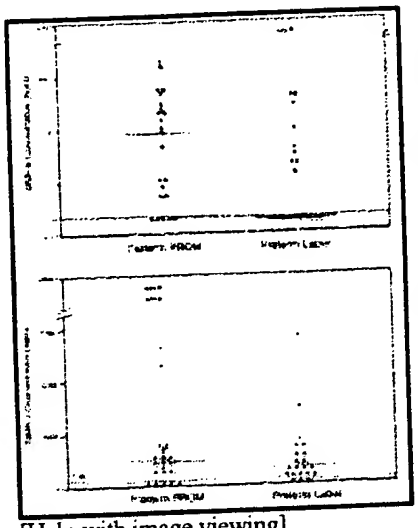

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Figure 2. MMP-9 and TIMP-1 concentrations in amniotic fluid in preterm gestation. Interrupted horizontal line, Sensitivity of assay (0.4 ng/mL and 1.08 ng/mL, respectively). Preterm premature rupture of membranes was associated with increase in amniotic fluid concentrations of MMP-9 and TIMP-1. MMP-9: Preterm premature rupture of membranes, median 7.6 ng/mL, range <0.4 to 230.8, versus preterm labor, median <0.4 ng/mL, range <0.4 to 1650, P = .06. TIMP-1: Preterm premature rupture of membranes, median 996.9 ng/mL, range <1.08 to 12,640, versus preterm labor, median 736.4 ng/mL, range <1.08 to 5795.8, P = .8). Wilcoxon test for censored observations.

Amniotic fluid MMP-9 concentrations were significantly higher in preterm gestations with microbial invasion of the amniotic cavity (premature rupture of membranes or intact membranes) than in those with sterile fluid (Figure 3). Moreover, women with preterm premature rupture of membranes and microbial invasion had a 3-fold higher median concentration of MMP-9 than those with microbial invasion and intact membranes. Amniotic fluid concentrations of TIMP-1 were higher when microbial invasion was associated with preterm labor and intact membranes than in preterm premature rupture of the membranes (Figure 3).

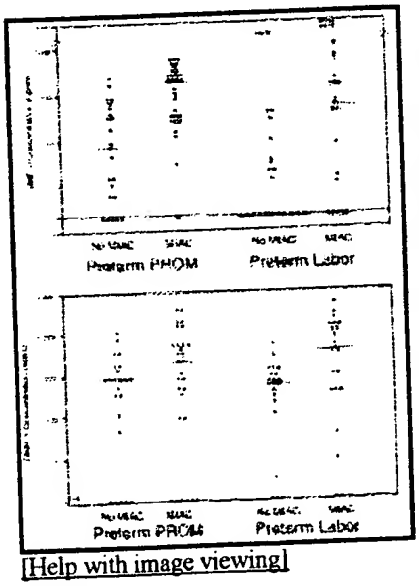

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Figure 3. MMP-9 and TIMP-1 concentrations in amniotic fluid in preterm women. Interrupted horizontal line, Sensitivity of assay (0.4 ng/mL and 1.08 ng/mL, respectively). Microbial invasion of amniotic cavity was associated with increased concentrations of amniotic fluid MMP-9 regardless of membrane status (preterm labor: microbial invasion, median 54.5 ng/mL, range <0.4 to 3910, versus no microbial invasion, median <0.4 ng/mL, range <0.4 to 1650, P < .01; preterm premature rupture of membranes: microbial invasion, median 179.8 ng/mL, range <0.4 to 611, versus no microbial invasion, median 7.6 ng/mL, range <0.4 to 230.81, P < .001). Microbial invasion of the amniotic cavity was associated with increased concentrations of amniotic fluid TIMP-1 in women with preterm labor and intact membranes but not in women with preterm premature rupture of membranes (preterm labor: microbial invasion, median 3786.8 ng/mL, range <1.1 to 59,400, versus no microbial invasion, median 736.4 ng/mL, range <1.1 to 36,300, P < .05; preterm premature rupture of membranes: microbial invasion, median 2383.8

ng/mL, range <1.1 to 42,000, versus no microbial invasion, median 996.9 ng/mL, range <1.1 to 12,530, P > .05). Wilcoxon test for censored observations.

A significant correlation was found between MMP-9 enzymatic activity and MMP-9 immunoreactivity (Spearman's rho, r = 0.45; P < .001). Because gelatin zymography provides only an approximate quantitation of the enzyme activity in amniotic fluid, [22,23] immunoreactive concentrations were chosen to explore differences among groups. Figure 4 shows a gelatin zymogram that depicts the differences in MMP-9 gelatinolytic activity in amniotic fluid from patients with preterm labor and preterm premature rupture of membranes. The amniotic fluid selected for display in this zymogram represents samples at, and close to, the median values in each group as documented by immunoassay and zymography.

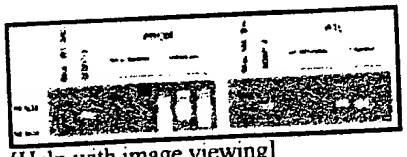

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Figure 4. Gelatin zymogram of MMP-9 activity in amniotic fluid. Microbial invasion of amniotic cavity was associated with increased activity of amniotic fluid MMP-9 regardless of membrane status. Patients with preterm premature rupture of membranes and microbial invasion had higher amniotic fluid MMP-9 activity than women with preterm labor, intact membranes, and microbial invasion.

The most common microbial isolate from women with preterm labor and intact membranes was Ureaplasma urealyticum (21 cases). Three patients had >1 microorganism isolated from the amniotic fluid. Similarly, the most frequent organism isolated from patients with preterm premature rupture of the membranes was U urealyticum (n = 14). Polymicrobial infection was detected in 15 cases (2 micro-organisms in 12 patients and 3 micro-organisms in the other 3 patients). None of the patients who were delivered at term had a positive amniotic fluid culture.

Comment

Our results demonstrate increased amniotic fluid MMP-9 availability in patients with rupture of membranes (term and preterm) and microbial invasion of the amniotic cavity in preterm gestations regardless of membrane status. It is noteworthy that the median MMP-9 amniotic fluid concentration in patients with preterm premature rupture of the membranes was 2-fold higher than that of patients with premature rupture of membranes at term. These observations could be interpreted as suggesting that a higher bioavailability of MMP-9 is required to degrade extracellular matrix associated with premature rupture of the membranes in the preterm gestation than in term pregnancy.

Collagen provides the basic framework of fetal membranes and is

responsible for most of their tensile strength. The main forms of collagen in chorioamniotic membranes are types I, III, IV, and V. [8] MMP-9 is capable of degrading collagen types IV and V. Type IV collagen forms the backbone onto which other basement membrane components are anchored and additionally is a major component of the spongy layer of the fetal membranes. [8] Striking changes in the spongy layer have been described histologically at the site of membrane rupture. Together with the documentation of delaminating of amnion epithelial cells from the basement membrane before the onset of spontaneous labor, [23] these observations suggest that degradation of type IV collagen may be an important step before membrane rupture. Our observations are consistent with those of McLaren et al, [24] who reported increased MMP-9 activity in the membranes in close proximity to the cervix where the morphologic changes associated with the development of the zone of altered morphologic characteristics has been described.

It may be argued that, because collagen type I forms the major component of amniochorion connective tissue, [8] degradation of this form of collagen is required for membrane rupture. MMP-1 is one of the matrix metalloproteinases capable of cleaving collagen types I and III. We have found that amniotic fluid concentrations of MMP-1 are not increased in the setting of premature rupture of the membranes either in preterm or term gestations (unpublished observations). This may be due to the fact that MMP-1 is tightly bound to the extracellular matrix and therefore amniotic fluid concentrations may not reflect tissue levels and activity. [21] Alternatively, degradation of types I and III collagen could be due to the activity of collagenases other than type I (eg, matrix metalloproteinase-8 and matrix metalloproteinase-13). Gelatinases such as MMP-9 may be required to digest the denatured collagen fragments (gelatin) produced by the action of MMP-1 on collagen type I. TIMP-1 and TIMP-2 are both normal constituents of human fetal membranes. [20] Inhibition of matrix metalloproteinase activity by tissue inhibitors occurs in a 1:1 stoichiometric relationship. TIMP-1 and TIMP-2 are present and may be involved in physiologic remodeling of the fetal membranes. The induction of MMP-9 in the context of infection may also play a role in membrane rupture. The observation in this study that microbial invasion of the amniotic cavity was associated with a strikingly increased availability of MMP-9 is in agreement with a previous report by Romero et al. [19] Although other investigators have invoked a role for proteases of microbial origin in the mechanisms responsible for membrane rupture, there is no empiric evidence to date that their activity is increased in vivo in cases of preterm premature rupture of the membranes associated with intrauterine infection. [7] The findings reported herein support the observations of Fortunato et al, [20] who documented induction of MMP-9 messenger ribonucleic acid in human fetal membranes in response to bacterial toxins. Our findings provide evidence for the participation of the host in this pathologic process. Microorganisms could stimulate resident macrophages to produce proinflammatory cytokines, which in turn would induce MMP-9 gene expression and lead to

secretion and activation of this enzyme and degradation of the extracellular matrix responsible for the integrity of the chorioamniotic membranes.

In the context of intrauterine infection, MMP-9 concentrations were significantly higher in patients with preterm premature rupture of the membranes than in those with preterm labor with intact membranes. This observation provides support for the hypothesis that the nature of the host response to intrauterine infection determines the clinical presentation. [25] If the production of uterotonic agents (eg, prostaglandins) and expression of the appropriate receptors in myometrium is the predominant response, increased uterine contractility (eg, preterm labor) will be the presenting symptom. On the other hand, if activation of matrix degrading enzymes is the predominant host response, then rupture of membranes will be the clinical manifestation of this pathologic process. Although coordination of these 2 components of the common terminal pathway of parturition frequently occurs, lack of coordination is also a clinical reality. Further research is required to determine why and how the host selects either a coordinated or a dys-synchronous activation of the 2 components of parturition to deal with the challenge of infection.

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